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## (54) USE OF THIOL REDOX PROTEINS FOR REDUCING DISULFIDE BONDS

VERWENDUNG VON THIOLGRUPPEN ENTHALTENDEN REDOX-PROTEINEN ZUR REDUKTION  
VON DISULFIDBINDUNGENUTILISATION DE PROTEINES D'OXYDOREDUCTION A BASE DE THIOL POUR REDUIRE DES  
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Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

**Description****CROSS-REFERENCE**

5 This application is a continuation-in-part of application Serial No. 07/776,109, filed October 12, 1991, and of application Serial No. 07/935,002, filed August 25, 1992 which is a continuation-in-part application of Serial No. 07/776,109, filed October 12, 1991.

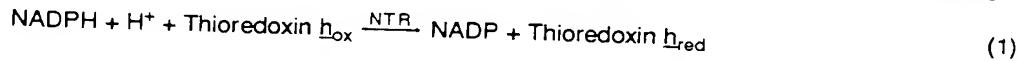
**FIELD OF THE INVENTION**

10 The present invention relates to the use of thiol redox proteins to reduce seed protein such as cereal proteins, enzyme inhibitor proteins, venom toxin proteins and the intramolecular disulfide bonds of certain other proteins. More particularly, the invention involves use of thioredoxin and glutaredoxin to reduce gliadins, glutenins, albumins and globulins to improve the characteristics of dough and baked goods and create new doughs and to reduce cystine containing 15 proteins such as amylase and trypsin inhibitors so as to improve the quality of feed and cereal products. Additionally, the invention involves the isolation of a novel protein that inhibits pullulanase and the reduction of that novel protein by thiol redox proteins. The invention further involves the reduction by thioredoxin of 2S albumin proteins characteristic of oil-storing seeds. Also, in particularly the invention involves the use of reduced thiol redox agents to inactivate snake 20 neurotoxins and certain insect and scorpion venom toxins *in vitro* and to treat the corresponding toxicities in individuals.

20 This invention was made with government support under Grant Contract Nos. DCB 8825980 and DMB 88-15980 awarded by the National Science Foundation. The United States Government has certain rights in this invention.

**BACKGROUND OF THE INVENTION**

25 Chloroplasts contain a ferredoxin/thioredoxin system comprised of ferredoxin, ferredoxin-thioredoxin reductase and thioredoxins *f* and *m* that links light to the regulation of enzymes of photosynthesis (Buchanan, B.B. (1991) "Regulation of CO<sub>2</sub> assimilation in oxygenic photosynthesis: The ferredoxin/thioredoxin system. Perspective on its discovery, present status and future development", *Arch. Biochem. Biophys.* **288**:1-9; Scheibe, R. (1991), "Redox-modulation of 30 chloroplast enzymes. A common principle for individual control", *Plant Physiol.* **96**:1-3). Several studies have shown that plants also contain a system, analogous to the one established for animals and most microorganisms, in which thioredoxin (*h*-type) is reduced by NADPH and the enzyme, NADP-thioredoxin reductase (NTR) according to the following:



35 (Florence F.J., et al. (1988), *Arch. Biochem. Biophys.* **266**:496-507; Johnson, T.C., et al. (1987), *Plant Physiol.* **85**:446-451; Suske, G., et al. (1979), *Z. Naturforsch. C* **34**:214-221). Current evidence suggests that the NADP/thioredoxin system is widely distributed in plant tissues and is housed in the mitochondria, endoplasmic reticulum and cytosol (Bodenstein-Lang, J., et al. (1989), *FEBS Lett.* **258**:22-26; Marcus, F., et al. (1991), *Arch. Biochem. Biophys.* **287**:195-198).

40 Thioredoxin *h* is also known to reductively activate cytosolic enzyme of carbohydrate metabolism, pyrophosphate fructose-6-P, 1-phosphotransferase or PFP (Kiss, F., et al. (1991), *Arch. Biochem. Biophys.* **287**:337-340).

The seed is the only tissue for which the NADP/thioredoxin system has been ascribed physiological activity in plants. Also, thioredoxin *h* has been shown to reduce thionins in the laboratory (Johnson, T.C., et al. (1987), *Plant Physiol.* **85**:446-451). Thionins are soluble cereal seed proteins, rich in cystine. In the Johnson, et al. investigation, wheat 45 purothionin was experimentally reduced by NADPH via NADP-thioredoxin reductase (NTR) and thioredoxin *h* according to Eqs. 2 and 3.



50 Cereal seeds such as wheat, rye, barley, corn, millet, sorghum and rice contain four major seed protein groups. These four groups are the albumins, globulins, gliadins and the glutenins or corresponding proteins. The thionins belong to the albumin group or fraction. Presently, wheat and rye are the only two cereals from which gluten or dough 55 has been formed. Gluten is a tenacious elastic and rubbery protein complex that gives cohesiveness to dough. Gluten is composed mostly of the gliadin and glutenin proteins. It is formed when rye or wheat dough is washed with water. It is the gluten that gives bread dough its elastic type quality. Flour from other major crop cereals barley, corn, sorghum, oat, millet and rice and also from the plant, soybean do not yield a gluten-like network under the conditions used for

wheat and rye.

Glutenins and gliadins are cystine containing seed storage proteins and are insoluble. Storage proteins are proteins in the seed which are broken down during germination and used by the germinating seedling to grow and develop. Prolamines are the storage proteins in grains other than wheat that correspond to gliadins while the glutelins are the storage proteins in grains other than wheat that correspond to glutenins. The wheat storage proteins account for up to 5 80% of the total seed protein (Kasarda, D.D., et al. (1976), Adv. Cer. Sci. Tech. 1:158-236; and Osborne, T.B., et al. (1893), Amer. Chem. J. 15:392-471). Glutenins and gliadins are considered important in the formation of dough and therefore the quality of bread. It has been shown from *in vitro* experiments that the solubility of seed storage proteins is increased on reduction (Shewry, P.R., et al. (1985), Adv. Cer. Sci. Tech. 7:1-83). However, previously, reduction of 10 glutenins and gliadins was thought to lower dough quality rather than to improve it (Dahle, L.K., et al. (1966), Cereal Chem. 43:682-688). This is probably because the non-specific reduction with chemical reducing agents caused the weakening of the dough.

The "Straight Dough" and the "Pre-Ferment" methods are two major conventional methods for the manufacture of dough and subsequent yeast raised bread products.

15 For the Straight Dough method, all of the flour, water or other liquid, and other dough ingredients which may include, but are not limited to yeast, grains, salt, shortening, sugar, yeast nutrients, dough conditioners, and preservatives are blended to form a dough and are mixed to partial or full development. The resulting dough may be allowed to ferment for a period of time depending upon specific process or desired end-product characteristics.

20 The next step in the process is the mechanical or manual division of the dough into appropriate size pieces of sufficient weight to ensure achieving the targeted net weight after baking, cooling, and slicing. The dough pieces are often then rounded and allowed to rest (Intermediate Proof) for varying lengths of time. This allows the dough to "relax" prior to sheeting and molding preparations. The time generally ranges from 5-15 minutes, but may vary considerably depending on specific processing requirements and formulations. The dough pieces are then mechanically or manually 25 formed into an appropriate shape are then usually given a final "proof" prior to baking. The dough pieces are then baked at various times, temperatures, and steam conditions in order to achieve the desired end product.

30 In the Pre-Ferment method, yeast is combined with other ingredients and allowed to ferment for varying lengths of time prior to final mixing of the bread or roll dough. Baker's terms for these systems include "Water Brew", "Liquid Ferment", "Liquid Sponge", and "Sponge/Dough". A percentage of flour ranging from 0-100% is combined with the other 35 ingredients which may include but are not limited to water, yeast, yeast nutrients and dough conditioners and allowed to ferment under controlled or ambient conditions for a period of time. Typical times range from 1-5 hours. The ferment may then be used as is, or chilled and stored in bulk tanks or troughs for later use. The remaining ingredients are added (flour, characterizing ingredients, additional additives, additional water, etc.) and the dough is mixed to partial or full development.

35 The dough is then allowed to ferment for varying time periods. Typically, as some fermentation has taken place prior to the addition of the remaining ingredients, the time required is minimal (i.e., 10-20 min.), however, variations are seen depending upon equipment and product type. Following the second fermentation step, the dough is then treated as in the Straight Dough Method.

40 As used herein the term "dough mixture" describes a mixture that minimally comprises a flour or meal and a liquid, such as milk or water.

45 As used herein the term "dough" describes an elastic, pliable protein network mixture that minimally comprises a flour, or meal and a liquid, such as milk or water.

As used herein the term "dough ingredient" may include, but is not exclusive of, any of the following ingredients: flour, water or other liquid, grain, yeast, sponge, salt, shortening, sugar, yeast nutrients, dough conditioners and preservatives.

50 As used herein, the term "baked good" includes but is not exclusive of all bread types, including yeast-leavened and chemically-leavened and white and variety breads and rolls, english muffins, cakes and cookies, confectionery coatings, crackers, doughnuts and other sweet pastry goods, pie and pizza crusts, pretzels, pita and other flat breads, tortillas, pasta products, and refrigerated and frozen dough products.

While thioredoxin has been used to reduce albumins in flour, thiol redox proteins have not been used to reduce 55 glutenins and gliadins nor other water insoluble storage proteins, nor to improve the quality of dough and baked goods. Thiol redox proteins have also not been used to improve the quality of gluten thereby enhancing its value nor to prepare dough from crop cereals such as barley, corn, sorghum, oat, millet and rice or from soybean flour.

DE-2 309 254 discloses a process for making leavened bread comprising the treatment of dough with a mixture of aminoacids selected from the group consisting of cystein, glutathione and mixture thereof, ascorbic acid, dried whey and dried soya protein.

The reducing agents used in this composition, i.e. cystein and glutathione, are monothiols, and not dithiols. Moreover this patent teaches clearly that the reducing agents do not act alone, but that it is the entire additive compositions that is responsible for the effect on the dough.

KOBREHEL et al. (1991, *J. Biol. Chem.*, 266, n°24, 161 35 161 40) describe the reduction by thioredoxin of  $\alpha$ -amylase and trypsin inhibitor proteins. But, this document does not disclose the reduction of components of gluten, such as gliadins or glutenins.

Many cereal seeds also contain proteins that have been shown to act as inhibitors of enzymes from foreign sources. It has been suggested that these enzyme inhibitors may afford protection against certain deleterious organisms (Garcia-OLMEDO, F. et al. (1987), *Oxford Surveys of Plant Molecular and Cell Biology* 4: 275-335; Birk, Y. (1976), *Meth. Enzymol.* 45:695-739, and Laskowski, M., Jr., et al. (1980), *Ann. Rev. Biochem.* 49:593-626). Two such type enzyme inhibitors are amylase inhibitors and trypsin inhibitors. Furthermore, there is evidence that a barley protein inhibitor (not tested in this study) inhibits an  $\alpha$ -amylase from the same source (Weselake, R.J. et al. (1983), *Plant Physiol.* 72:809-812). Unfortunately, the inhibitor protein often causes undesirable effects in certain food products. The trypsin inhibitors in soybeans, notably the Kunitz trypsin inhibitor (KTI) and Bowman-Birk trypsin inhibitor (BBTI) proteins, must first be inactivated before any soybean product can be ingested by humans or domestic animals. It is known that these two inhibitor proteins become ineffective as trypsin inhibitors when reduced chemically by sodium borohydride (Birk, Y. (1985), *Int. J. Peptide Protein Res.* 25:113-131, and Birk, Y. (1976), *Meth. Enzymol.* 45: 695-739). These inhibitors like other proteins that inhibit proteases contain intramolecular disulfides and are usually stable to inactivation by heat and proteolysis (Birk (1976)), *supra*; Garcia-Olmedo, et al. (1987), *supra*, and Ryan (1980). Currently, to minimize the adverse effects caused by the inhibitors these soybean trypsin inhibitors and other trypsin inhibitors in animal and human food products are being treated by exposing the food to high temperatures. The heat treatment, however, does not fully eliminate inhibitor activity. Further, this process is not only expensive but it also destroys many of the other proteins which have important nutritional value. For example, while 30 min at 120°C leads to complete inactivation of the BBTI of soy flour, about 20% of the original KTI activity remains (Friedman, et al., 1991). The prolonged or higher temperature treatments required for full inactivation of inhibitors results in destruction of amino acids such as cystine, arginine, and lysine (Chae, et al., 1984; Skrede and Krogdahl, 1985).

There are also several industrial processes which require  $\alpha$ -amylase activity. One example is the malting of barley which requires active  $\alpha$ -amylase. Inactivation of inhibitors such as the barley amylase/subtilisin (asi) inhibitor and its equivalent in other cereals by thiol redox protein reduction would enable  $\alpha$ -amylases to become fully active sooner than with present procedures, thereby shortening time for malting or similar processes.

Thiol redox proteins have also not previously been used to inactivate trypsin or amylase inhibitor proteins. The reduction of trypsin inhibitors such as the Kunitz and Bowman-Birk inhibitor proteins decreases their inhibitory effects (Birk, Y. (1985), *Int. J. Peptide Protein Res.* 25:113-131). A thiol redox protein linked reduction of the inhibitors in soybean products designed for consumption by humans and domestic animals would require no heat or lower heat than is presently required for protein denaturation, thereby cutting the costs of denaturation and improving the quality of the soy protein. Also a physiological reductant, a so-called clean additive (*i.e.*, an additive free from ingredients viewed as "harmful chemicals") is highly desirable since the food industry is searching for alternatives to chemical additives. Further the ability to selectively reduce the major wheat and seed storage proteins which are important for flour quality (*e.g.*, the gliadins and the glutenins) in a controlled manner by a physiological reductant such as thiol redox protein would be useful in the baking industry for improving the characteristics of the doughs from wheat and rye and for creating doughs from other grain flours such as cereal flours or from cassava or soybean flour.

The family of 2S albumin proteins characteristic of oil-storing seeds such as castor bean and Brazil nut (Kreis, et al. 1989; Youle and Huang, 1981) which are housed within protein bodies in the seed endosperm or cotyledons (Ashton, et al. 1976; Weber, et al. 1980), typically consist of dissimilar subunits connected by two intermolecular disulfide bonds -- one subunit of 7 to 9 kDa and the other of 3 to 4 kDa. The large subunit contains two intramolecular disulfide groups, the small subunit contains none. The intramolecular disulfides of the large 2S large subunit show homology with those of the soybean Bowman-Birk inhibitor (Kreis, et al. 1989) but nothing is known of the ability of 2S proteins to undergo reduction under physiological conditions.

These 2S albumin proteins are rich in methionine. Recently transgenic soybeans which produce Brazil nut 2S protein have been generated. Reduction of the 2S protein in such soybeans could enhance the integration of the soy proteins into a dough network resulting in a soybread rich in methionine. In addition, these 2S proteins are often allergens. Reduction of the 2S protein would result in the cessation of its allergic activity.

Pullulanase ("debranching enzyme") is an enzyme that breaks down the starch of the endosperm of cereal seeds by hydrolytically cleaving  $\alpha$ -1,6 bonds. Pullulanase is an enzyme fundamental to the brewing and baking industries. Pullulanase is required to break down starch in malting and in certain baking procedures carried out in the absence of added sugars or other carbohydrates. Obtaining adequate pullulanase activity is a problem especially in the malting industry. It has been known for some time that dithiothreitol (DTT, a chemical reductant for thioredoxin) activates pullulanase of cereal preparations (*e.g.*, barley, oat and rice flours). A method for adequately activating or increasing the activity of pullulanase with a physiologically acceptable system, could lead to more rapid malting methods and, owing to increased sugar availability, to alcoholic beverages such as beers with enhanced alcoholic content.

Death and permanent injury resulting from snake bites are serious problems in many African, Asian and South

American countries and also a major concern in several southern and western areas of the United States. Venoms from snakes are characterized by active protein components (generally several) that contain disulfide (S-S) bridges located in intramolecular (intrachain) cystines and in some cases in intermolecular (interchain) cystines. The position of the cystine within a given toxin group is highly conserved. The importance of intramolecular S-S groups to toxicity is evident from reports showing that reduction of these groups leads to a loss of toxicity in mice (Yang, C.C. (1967) *Biochim. Biophys. Acta.* **133**:346-355; Howard, B.D., et al. (1977) *Biochemistry* **16**:122-125). The neurotoxins of snake venom are proteins that alter the release of neurotransmitter from motor nerve terminals and can be presynaptic or postsynaptic. Common symptoms observed in individuals suffering from snake venom neurotoxicity include swelling, edema and pain, fainting or dizziness, tingling or numbing of affected part, convulsions, muscle contractions, renal failure, in addition to long-term necrosis and general weakening of the individual, etc.

The presynaptic neurotoxins are classified into two groups. The first group, the  $\beta$ -neurotoxins, include three different classes of proteins, each having a phospholipase A<sub>2</sub> component that shows a high degree of conservation. The proteins responsible for the phospholipase A<sub>2</sub> activity have from 6 to 7 disulfide bridges. Members of the  $\beta$ -neurotoxin group are either single chain (e.g., caudotoxin, notexin and agkistrodotoxin) or multichain (e.g., crototoxin, ceruleotoxin and *Vipera* toxin).  $\beta$ -bungarotoxin, which is made up of two subunits, constitutes a third group. One of these subunits is homologous to the Kunitz-type proteinase inhibitor from mammalian pancreas. The multichain  $\beta$ -neurotoxins have their protein components linked ionically whereas the two subunits of  $\beta$ -bungarotoxin are linked covalently by an intermolecular disulfide. The B chain subunit of  $\beta$ -bungarotoxin, which is also homologous to the Kunitz-type proteinase inhibitor from mammalian pancreas, has 3 disulfide bonds.

The second presynaptic toxin group, the facilitatory neurotoxins, is devoid of enzymatic activity and has two subgroups. The first subgroup, the dendrotoxins, has a single polypeptide sequence of 57 to 60 amino acids that is homologous with Kunitz-type trypsin inhibitors from mammalian pancreas and blocks voltage sensitive potassium channels. The second subgroup, such as the fasciculins (e.g., fasciculin 1 and fasciculin 2) are cholinesterase inhibitors and have not been otherwise extensively studied.

The postsynaptic neurotoxins are classified either as long or short neurotoxins. Each type contains S-S groups, but the peptide is unique and does not resemble either phospholipase A<sub>2</sub> or the Kunitz or Kunitz-type inhibitor protein. The short neurotoxins (e.g., erabutoxin a and erabutoxin b) are 60 to 62 amino acid residues long with 4 intramolecular disulfide bonds. The long neurotoxins (e.g.,  $\alpha$ -bungarotoxin and  $\alpha$ -cobratoxin) contain from 65 to 74 residues and 5 intramolecular disulfide bonds. Another type of toxins, the cytotoxins, acts postsynaptically but its mode of toxicity is ill defined. These cytotoxins show obscure pharmacological effects, e.g., hemolysis, cytolysis and muscle depolarization. They are less toxic than the neurotoxins. The cytotoxins usually contain 60 amino acids and have 4 intramolecular disulfide bonds. The snake venom neurotoxins all have multiple intramolecular disulfide bonds.

The current snake antitoxins used to treat poisonous snake bites following first aid treatment in individuals primarily involve intravenous injection of antivenom prepared in horses. Although it is not known how long after envenomation the antivenom can be administered and be effective, its use is recommended up to 24 hours. Antivenom treatment is generally accompanied by administration of intravenous fluids such as plasma, albumin, platelets or specific clotting factors. In addition, supporting medicines are often given, for example, antibiotics, antihistamines, antitetanus agents, analgesics and sedatives. In some cases, general treatment measures are taken to minimize shock, renal failure and respiratory failure. Other than administering calcium-EDTA in the vicinity of the bite and excising the wound area, there are no known means of localized treatment that result in toxin neutralization and prevention of toxic uptake into the blood stream. Even these localized treatments are of questionable significance and are usually reserved for individuals sensitive to horse serum (Russell, F.E. (1983) *Snake Venom Poisoning*, Schollum International, Inc. Great Neck, NY).

The term "individual" as defined herein refers to an animal or a human. Most of the antivenoms in current use are problematic in that they can produce harmful side effects in addition to allergic reactions in patients sensitive to horse serum (up to 5% of the patients). Nonallergic reactions include pyrogenic shock, and complement depletion (Chippaur, J.-P., et al. (1991) *Reptile Venoms and Toxins*, A.T. Tu, ed., Marcel Dekker, Inc., pp. 529-555).

It has been shown that thioredoxin, in the presence of NADPH and thioredoxin reductase reduces the bacterial neurotoxins tetanus and botulinum A *in vitro* (Schiavo, G., et al. (1990) *Infection and Immunity* **58**:4136-4141; Kistner, A., et al. (1992) *Naunyn-Schmiedeberg's Arch Pharmacol* **345**:227-234). Thioredoxin was effective in reducing the interchain disulfide link of tetanus toxin and such reduced tetanus toxin was no longer neurotoxic (Schiavo, et al., *supra*). However, reduction of the interchain disulfide of botulinum A toxin by thioredoxin was reported to be much more sluggish (Kistner, et al., *supra*). In contrast to the snake neurotoxin studied in the course of this invention, the tetanus research group (Schiavo, et al., *supra*) found no evidence in the work done with the tetanus toxin that reduced thioredoxin reduced toxin intrachain disulfide bonds. There was also no evidence that thioredoxin reduced intrachain disulfides in the work done with botulinum A. The tetanus and botulinum A toxins are significantly different proteins from the snake neurotoxins in that the latter (1) have a low molecular weight; (2) are rich in intramolecular disulfide bonds; (3) are resistant to trypsin and other animal proteases; (4) are active without enzymatic modification, e.g., proteolytic

cleavage; (5) in many cases show homology to animal proteins, such as phospholipase A<sub>2</sub> and Kunitz-type proteases; (6) in most cases lack intermolecular disulfide bonds, and (7) are stable to agents such as heat and proteases.

Reductive inactivation of snake toxins *in vitro* by incubation with 1%  $\beta$ -mercaptoethanol for 6 hours and incubation with 8M urea plus 300mM  $\beta$ -mercaptoethanol has been reported in the literature (Howard, B.D., et al. (1977) *Biochemistry* **16**:122-125; Yang, C.C. (1967) *Biochim. Biophys. Acta* **133**:346-355). These conditions, however, are far from physiological. As defined herein the term "inactivation" with respect to a toxin protein means that the toxin is no longer biologically active *in vitro*, in that the toxin is unable to link to a receptor. Also as used herein, "detoxification" is an extension of the term "inactivation" and means that the toxin has been neutralized in an individual as determined by animal toxicity tests.

Bee venom is a complex mixture with at least 40 individual components, that include major components as melittin and phospholipase A<sub>2</sub>, representing respectively 50% and 12% of the total weight of the venom, and minor components such as small proteins and peptides, enzymes, amines, and amino acids.

Melittin is a polypeptide consisting of 26 amino acids with a molecular weight of 2840. It does not contain a disulfide bridge. Owing to its high affinity for the lipid-water interphase, the protein permeates the phospholipid bilayer of the cell membranes, disturbing its organized structure. Melittin is not by itself a toxin but it alters the structure of membranes and thereby increases the hydrolytic activity of phospholipase A<sub>2</sub>, the other major component and the major allergen present in the venom.

Bee venom phospholipase A<sub>2</sub> is a single polypeptide chain of 128 amino acids, is cross-linked by four disulfide bridges, and contains carbohydrate. The main toxic effect of the bee venom is due to the strong hydrolytic activity of phospholipase A<sub>2</sub> achieved in association with melittin.

The other toxic proteins in bee venom have a low molecular weight and contain at least two disulfide bridges that seem to play an important structural role. Included are a protease inhibitor (63-65 amino acids), MCD or 401-peptide (22 amino acids) and apamin (18 amino acids).

Although there are thousands of species of bees, only the honey bee, *Apis mellifera*, is a significant cause of allergic reactions. The response ranges from local discomfort to systemic reactions such as shock, hypotension, dyspnea, loss of consciousness, wheezing and/or chest tightness that can result in death. The only treatment that is used in these cases is the injection of epinephrine.

The treatment of bee stings is important not only for individuals with allergic reactions. The "killer" or Africanized bee, a variety of honey bee is much more aggressive than European honey bees and represents a danger in both South and North America. While the lethality of the venom from the Africanized and European bees appears to be the same (Schumacher, M. I., et al. (1989) *Nature* **337**:413), the behaviour pattern of the hive is completely different. It was reported that Africanized bees respond to colony disturbance more quickly, in greater numbers and with more stinging (Collins, A. M., et al. (1982) *Science* **218**:72-74). A mass attack by Africanized bees may produce thousands of stings on one individual and cause death. The "killer" bees appeared as a result of the interbreeding between the African bee (*Apis mellifera scutellata*) and the European bee (*Apis mellifera mellifera*). African bees were introduced in 1956 into Brazil with the aim of improving honey production being a more tropically adapted bee. Africanized bees have moved from South America to North America, and they have been reported in Texas and Florida.

In some areas of the world such as Mexico, Brazil, North Africa and the Middle East, scorpions present a life hazard to humans. However, only the scorpions of family Buthidae (genera, *Androctonus*, *Buthus*, *Centruroides*, *Leiurus* and *Tityus*) are toxic for humans. The chemical composition of the scorpion venom is not as complex as snake or bee venom. Scorpion venom contains mucopolysaccharides, small amounts of hyaluronidase and phospholipase, low molecular-weight molecules, protease inhibitors, histamine releasers and neurotoxins, such as serotonin. The neurotoxins affect voltage-sensitive ionic channels in the neuromuscular junction. The neurotoxins are basic polypeptides with three to four disulfide bridges and can be classified in two groups: peptides with from 61 to 70 amino acids, that block sodium channel, and peptides with from 36 to 39 amino acids, that block potassium channel. The reduction of disulfide bridges on the neurotoxins by nonphysiological reductants such as DTT or  $\beta$ -mercaptoethanol (Watt, D. D., et al. (1972) *Toxicon* **10**:173-181) lead to loss of their toxicity.

Symptoms of animals stung by poisonous scorpions include hyperexcitability, dyspnea, convulsions, paralysis and death. At present, antivenin is the only antidote for scorpion stings. The availability of the venom is a major problem in the production of antivenin. Unlike snake venom, scorpion venom is very difficult to collect, because the yield of venom per specimen is limited and in some cases the storage of dried venom leads to modification of its toxicity. An additional problem in the production of antivenins is that the neurotoxins are very poor antigens.

The reductive inactivation of snake, bee and scorpion toxins under physiological conditions has never been reported nor has it been suggested that the thiol redox agents, such as reduced lipoic acid, DTT, or reduced thioredoxin could act as an antidote to these venoms in an individual.

SUMMARY OF THE INVENTION

It is an object herein to provide a method for reducing a non thionin cystine containing protein.

It is a second object herein to provide methods utilizing a thiol redox protein alone or in combination with a reductant or reduction system to reduce glutenins or gliadins present in flour or seeds.

It is also an object herein to provide methods using a thiol redox protein alone or in combination with a reductant or reduction system to improve dough strength and baked goods characteristics such as better crumb quality, softness of the baked good and higher loaf volume.

It is a further object herein to provide formulations containing a thiol redox protein useful in practicing such methods.

Still a further object herein is to provide a method for producing a dough from rice, corn, soybean, barley, oat, cassava, sorghum or millet flour.

Yet, another object is to provide a method for producing an improved gluten or for producing a gluten-like product from cereal grains other than wheat and rye.

Still another object herein is to provide yeast cells genetically engineered to express or overexpress thioredoxin.

Still yet another object herein is to provide yeast cells genetically engineered to express or overexpress NADP-thioredoxin reductase.

Still yet a further object herein is to provide a method for improving the quality of dough or a baked good using such genetically engineered yeast cells.

In accordance with the objects of the invention, methods are provided for improving dough characteristics comprising the steps of mixing a thiol redox protein with dough ingredients to form a dough and baking said dough.

The thiol redox proteins in use herein can include thioredoxin and glutaredoxin. The thioredoxin includes but is not exclusive of *E. coli* thioredoxin, thioredoxin h, f and m and animal thioredoxins. A reductant of thioredoxin used herein can include lipoic acid or a reduction system such as NADPH in combination with NADP thioredoxin reductase (NTR).

The reductant of glutaredoxin can include reduced glutathione in conjunction with the reduction system NADPH and glutathione reductase. NADPH can be replaced with an NADPH generator or generator composition such as one consisting of glucose 6-phosphate, NADP and glucose 6-phosphate dehydrogenase from a source such as yeast. The NADPH generator is added together with thioredoxin and NADP-thioredoxin reductase at the start of the dough making process.

It should be noted that the invention can also be practiced with cysteine containing proteins. The cysteines can first be oxidized and then reduced via thiol redox protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts a graph showing the effect of  $\alpha$ -amylase protein inhibitors on activation of NADP-Malate Dehydrogenase in the presence of DTT-reduced Thioredoxin h.

Fig. 2 is a graph showing the effect of  $\alpha$ -amylase Inhibitor Concentration on NADP-Malate Dehydrogenase Activation by  $\alpha$ -amylase Inhibitors.

Fig. 3 is a graph showing the effect of Thioredoxin h Concentration on Activation of NADP-Malate dehydrogenase by DSG-1 or -2  $\alpha$ -Amylase Inhibitors.

Fig. 4 is a graph showing the effect of  $\alpha$ -Amylase Inhibitors on DTNB Reduction by the *E. coli* NADP/Thioredoxin System.

Fig. 5 is a graph showing the effect of purothionin  $\alpha$  and CM-1  $\alpha$ -Amylase Inhibitor from Bread Wheat on DTNB Reduction by the *E. coli* NADP/Thioredoxin System.

Fig. 6 is a photograph taken of an SDS polyacrylamide electrophoretic gel placed over a long UV wavelength light box showing the Thioredoxin-Linked Reduction of Soluble Sulfur Rich Seed Proteins: Durum Wheat  $\alpha$ -Amylase Inhibitor (DSG-1) and Bowman-Birk soybean Trypsin Inhibitor (BBTI).

Fig. 7 is a photograph taken of an SDS polyacrylamide electrophoretic gel placed over a long UV wavelength light box showing the Thioredoxin-Linked Reduction of Seed Proteins.

Fig. 8 is a photograph taken of an SDS-polyacrylamide electrophoretic gel placed over a fluorescent light box showing Thioredoxin-linked reduction of gliadins determined by an SDS-PAGE/mBBr procedure.

Fig. 9 is a photograph taken of an acidic-polyacrylamide electrophoretic gel placed over a fluorescent light box showing Thioredoxin-linked reduction of the different types of gliadins determined by an acid PAGE/mBBr procedure.

5 Fig. 10 is a photograph taken of an SDS-polyacrylamide electrophoretic gel placed over a fluorescent light box showing Thioredoxin-linked reduction of acid soluble glutenins determined by an SDS-PAGE/mBBr procedure.

10 Fig. 11A and Fig. 11B are graphs showing the protein content and the relative reduction of seed protein fractions during germination.

15 Fig. 12 is a bar graph showing reduction of principal thioredoxin-linked gliadins and glutenins during germination (compared with *in vivo* reduction).

20 Fig. 13 is a diagrammatic representation of the proposed role of thioredoxin in forming a protein network for bread and pasta.

25 Fig. 14 shows farinograms of treated and untreated medium quality flour; Fig. 14(a) is the farinogram of the medium quality flour; Fig. 14(b) is of the same flour following treatment with reduced glutathione, and Fig. 14(c) is of the medium quality flour after treatment with the NADP/thioredoxin system.

30 Fig. 15 shows farinograms of treated and untreated poor quality flour; Fig. 15(a) is a farinogram of the poor quality flour control; Fig. 15(b) is of the same flour after treatment with reduced glutathione, and Fig. 15(c) is of the poor quality flour after treatment with DTT, and Fig. 15(d) is of the poor quality flour after treatment with the NADP/thioredoxin system.

35 Fig. 16 shows farinograms of treated and untreated Apollo flour; Fig. 16(a) represents the untreated flour, and Fig. 16(b) represents the same flour treated with the NTS.

40 Fig. 17 shows farinograms of treated and untreated Apollo flour; Fig. 17(a) is a farinogram of the Apollo control; Fig. 17(b) is of the same flour after treatment with an NADPH generating system; Fig. 17(c) is of the Apollo flour after treatment with the same generating system plus NTR and thioredoxin.

45 Fig. 18 is a photograph showing a top view of a comparison between an Arbon loaf of bread made from thioredoxin-treated dough and an untreated control loaf.

50 Fig. 19 is a photograph showing a side elevational and partial top view of a comparison between a loaf made from thioredoxin-treated Arbon flour dough and an untreated control loaf.

55 Fig. 20 is a photograph showing a side elevational view of a comparison between a loaf made from a thioredoxin-treated Arbon flour dough and an untreated control loaf.

60 Fig. 21 is a photograph showing a top view of a comparison between a loaf made from a thioredoxin-treated Arbon flour dough and an untreated control loaf.

65 Fig. 22 is a photograph showing a side elevational and partial top view of a comparison between a loaf made from a thioredoxin-treated Arbon flour dough and an untreated control loaf.

70 Fig. 23 shows photographs comparing breads baked from thioredoxin treated and untreated doughs; Fig. 23(a) shows a comparison of loaves of bread made from treated and untreated arbon flour, and Fig. 23(b) shows a comparison among baked goods that were prepared from thioredoxin-treated and untreated corn, rice and sorghum flour.

75 Fig. 24 is a photograph showing a top and partial side view of a comparison between a loaf baked from a triticale flour dough treated with thioredoxin and a control loaf made from untreated triticale flour dough.

80 Fig. 25 is a photograph showing comparisons among baked goods that were prepared from thioredoxin-treated and untreated corn, rice and sorghum flour.

Fig. 26 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from oat flour.

5 Fig. 27 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from triticale flour.

Fig. 28 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from rye flour.

10 Fig. 29 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from barley flour.

15 Fig. 30 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of buffer, ethanol and myristate-extracted proteins from teff flour; Fig. 30(a) shows fluorescence and Fig. 30(b) shows the protein staining.

Fig. 31 is a photograph of an SDS polyacrylamide electrophoretic gel showing the effect of NTS vs. glutathione reductase on the reduction status of myristate-extracted proteins from corn, sorghum and rice.

20 Fig. 32 is a photograph of an SDS polyacrylamide electrophoretic gel showing the *in vivo* reduction status and thioredoxin-linked *in vitro* reduction of myristate-extracted proteins from corn, sorghum and rice.

Fig. 33 represents photographs of an SDS polyacrylamide electrophoretic gel showing the relative reduction of wheat glutenins by a yeast NADP/thioredoxin system.

25 Fig. 34 represents photographs of an SDS polyacrylamide electrophoretic gel showing the relative reduction of wheat gliadins by a yeast NADP/thioredoxin system.

Fig. 35 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of ethanol-extracted proteins from triticale flour.

30 Fig. 36 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of ethanol-extracted proteins from rye flour.

Fig. 37 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of ethanol-extracted proteins from oat flour.

35 Fig. 38 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of ethanol-extracted proteins from barley flour.

40 Fig. 39 represents photographs of an SDS polyacrylamide electrophoretic gels showing the extent of reduction of castor seed matrix and crystalloid proteins by various reductants.

Fig. 40 is a photograph of an SDS polyacrylamide electrophoretic gel showing the reduction specificity of 2S proteins.

45 Fig. 41 is a graph showing the separation of pullulanase inhibitor from pullulanase of barley malt by DE52 chromatography.

Fig. 42 is a graph showing the purification of pullulanase inhibitor of barley malt by CM32 chromatography at pH 4.6.

50 Fig. 43 is a graph showing the purification of pullulanase inhibitor of barley malt by CM32 chromatography at pH 4.0.

55 Fig. 44 is a graph showing the purification of pullulanase inhibitor of barley malt by Sephadex G-75 chromatography.

Fig. 45 represents photographs of SDS polyacrylamide electrophoretic gels showing the extent of reduction of bee venom proteins by various reductants.

5 Fig. 46 represents photographs of SDS polyacrylamide electrophoretic gels showing the extent of reduction of scorpion venom proteins by various reductants.

Fig. 47 represents photographs of SDS polyacrylamide electrophoretic gels showing the extent of reduction of snake venom proteins by various reductants.

10 Fig. 48 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of reduction of bee, scorpion and snake venom proteins with the NTS in the presence and absence of protease inhibitors.

15 Fig. 49 is a photograph of an SDS polyacrylamide electrophoretic gel showing the extent of reduction of erabutoxin b samples treated with different reductants.

Fig. 50 is a graph showing the activation of chloroplast NADP-malate dehydrogenase by erabutoxin b reduced with different thioredoxins compared to the activation of the dehydrogenase by a control lacking toxin.

20 Fig. 51 is a graph showing the effect of thioredoxin-linked reduction of  $\beta$ -bungarotoxin on  $\beta$ -bungarotoxin phospholipase A<sub>2</sub> activity.

Fig. 52 is a photograph of an SDS polyacrylamide electrophoretic gel showing the extent of reduction of  $\beta$ -bungarotoxin and  $\alpha$ -bungarotoxin samples with cellular reductants.

25 **DETAILED DESCRIPTION OF THE INVENTION**

In accordance with this detailed description, the following definitions and abbreviations apply:

CM -	certain bread wheat $\alpha$ -amylase inhibitors
DSG -	certain $\alpha$ -amylase inhibitors isolated from durum wheat
DTNB -	2'5'-dithiobis (2-nitrobenzoic acid)
NTR -	NADP-thioredoxin reductase
mBBr -	monobromobimane
NADP-MDH -	NADP-malate dehydrogenase
35 FBPase -	fructose-1,6-bisphosphatase
SDS -	sodium dodecyl sulfate
DTT -	dithiothreitol
Cereal -	millet, wheat, oat, barley, rice, sorghum, or rye
BBTI -	Bowman-Birk soybean trypsin inhibitor
40 KTI -	Kunitz soybean trypsin inhibitor
PAGE -	polyacrylamide gel electrophoresis
TCA -	trichloroacetic acid

45 **Enzyme Inhibitor Protein Experiments**

**Starting Materials**

**Materials**

50 Seeds of bread wheat Triticum aestivum L, cv. Talent) and durum wheat (Triticum durum Desf., cv. Mondur) were obtained from laboratory stocks.

**Reagents**

55 Chemicals and fine chemicals for enzyme assays and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co. and BioRad Laboratories, respectively. Monobromobimane (mBBr, tradename Thiolite) was purchased from Calbiochem. Other chemicals were obtained from commercial sources and were of the highest quality available.

Enzymes

Thioredoxin and NTR from *E. coli* were purchased from American Diagnostics, Inc. and were also isolated from cells transformed to overexpress each protein. The thioredoxin strain containing the recombinant plasmid, pFP1, was kindly provided by Dr. J.-P. Jacquot (de la Motte-Guery, F. et al. (1991) *Eur. J. Biochem.* 196: 287-294). The NTR strain containing the recombinant plasmid, pPMR21, was kindly provided by Drs. Marjorie Russel and Peter Model (Russel, M. et al. (1988) *J. Biol. Chem.* 263:9015-9019). The isolation procedure used for these proteins was as described in those studies with the following changes: cells were broken in a Ribi cell fractionator at 25,000 psi and NTR was purified as described by Florencio et al. (Florencio, F. J. et al. (1988) *Arch. Biochem. Biophys.* 266:496-507) without the red agarose step. Thioredoxin and NTR from *Saccharomyces cerevisiae* (baker's yeast type 1) were isolated by the procedure developed by Florencio, et al. for spinach leaves with the following changes: suspended cells [1 part cells:5 parts buffer (w/v)], were broken in a Ribi cell fractionator at 40,000 psi with three passes.

Thioredoxin *h* and NTR were isolated from wheat germ by the procedure developed for spinach leaves (Florencio, F.J., et al. (1988), *Arch. Biochem. Biophys.* 266:496-507). NADP-malate dehydrogenase (NADP-MDH) and fructose-1,6-bisphosphatase (FBPase) were purified from leaves of corn (Jacquot, J.-P., et al. (1981), *Plant Physiol.* 68:300-304) and spinach (Crawford, N.A., et al. (1989), *Arch. Biochem. Biophys.* 271:223-239) respectively. *E. coli* glutaredoxin and calf thymus thioredoxin were obtained from Professor A. Holmgren.

 $\alpha$ -Amylase and trypsin inhibitors

CM-1 protein was isolated from the albumin-globulin fraction of bread wheat flour as described in Kobrehel, K., et al. (1991), *Cereal Chem.* 68:1-6. A published procedure was also used for the isolation of DSG proteins (DSG-1 and DSG-2) from the glutenin fraction of durum wheat (Kobrehel, K. et al. (1989), *J. Sci. Food Agric.* 48:441-452). The CM-1, DSG-1 and DSG-2 proteins were homogeneous in SDS-polyacrylamide gel electrophoresis. Trypsin inhibitors were purchased from Sigma Chemical Co., except for the one from corn kernel which was from Fluka. In all cases, the commercial preparations showed a single protein component which migrated as expected in SDS-PAGE (Coomassie Blue stain), but in certain preparations, the band was not sharp.

Other proteins

Purothionin  $\alpha$  from bread wheat and purothionins  $\alpha$ -1 and  $\beta$  from durum wheat were kind gifts from Drs. D.D. Kasarda and B.L. Jones, respectively. The purothionin  $\alpha$  sample contained two members of the purothionin family when examined with SDS-polyacrylamide gel electrophoresis. The purothionin  $\alpha$ -1 and  $\beta$  samples were both homogeneous in SDS-polyacrylamide gel electrophoresis.

Routine Method StepsEnzyme activation assays

The NADP-MDH, FBPase, NTR and Thioredoxin *h* assay methods were according to Florencio, F.J., et al. (1988), *Arch. Biochem. Biophys.* 266:496-507 with slight modifications as indicated. For enzyme activation assays, the preincubation time was 20 min. unless specified otherwise.

mBBr fluorescent labeling and SDS-polyacrylamide gel electrophoresis analyses

Direct reduction of the proteins under study was determined by a modification of the method of Crawford, et al. (Crawford, N.A., et al. (1989), *Arch. Biochem. Biophys.* 271:223-239). The reaction was carried out in 100 mM potassium phosphate buffer, pH 7.1, containing 10 mM EDTA and 16% glycerol in a final volume of 0.1 ml. As indicated, 0.7  $\mu$ g (0.1  $\mu$ M) NTR and 1  $\mu$ g (0.8  $\mu$ M) thioredoxin (both routinely from *E. coli* were added to 70  $\mu$ l of the buffer solution containing 1 mM NADPH and 10  $\mu$ g (2 to 17  $\mu$ M) of target protein. When thioredoxin was reduced by dithiothreitol (DTT, 0.5 mM), NADPH and NTR were omitted. Assays with reduced glutathione were performed similarly, but at a final concentration of 1 mM. After incubation for 20 min, 100 nmoles of mBBr were added and the reaction was continued for another 15 min. To stop the reaction and derivatize excess mBBr, 10  $\mu$ l of 10% SDS and 10  $\mu$ l of 100 mM  $\beta$ -mercaptoethanol were added and the samples were then applied to the gels. In the case of reduction by glutaredoxin, the thioredoxin and NTR were replaced by 1  $\mu$ g (0.8  $\mu$ M) *E. coli* glutaredoxin, 1.4  $\mu$ g (0.14  $\mu$ M) glutathione reductase purified from spinach leaves (Florencio, F.J., et al. (1988), *Arch. Biochem. Biophys.* 266:496-507) and 1.5 mM NADPH was used.

Gels (17.5% w/v, 1.5 mm thickness) were prepared according to Laemmli (Laemmli, U.K. (1970), *Nature* 227:680-

685) and developed for 16 hr. at constant current (9 mA). Following electrophoresis, gels were placed in a solution of 40% methanol and 10% acetic acid, and soaked for 4 to 6 hours with several changes of the solution. Gels were then examined for fluorescent bands with near ultraviolet light and photographed (exposure time 25 sec) according to Crawford, *et al.* (Crawford, N.A., *et al.* (1989), *Arch. Biochem. Biophys.* 271:223-239). Finally, gels were stained with Coomassie Blue and destained as before (Crawford, N.A., *et al.* (1989), *Arch. Biochem. Biophys.* 271:223-239).

#### Quantification of labeled proteins

To obtain a quantitative indication of the extent of reduction of test proteins by the NADP/thioredoxin system, the intensities of their fluorescent bands seen in SDS-polyacrylamide gel electrophoresis were evaluated, using a modification of the procedure of Crawford, *et al.* (Crawford, N.A., *et al.* (1989), *Arch. Biochem. Biophys.* 271:223-239). The photographic negatives were scanned using a Pharmacia Ultrascan laser densitometer, and the area underneath the peaks was quantitated by comparison to a standard curve determined for each protein. For the latter determination, each protein (at concentrations ranging from 1 to 5  $\mu$ g) was reduced by heating for 3 min. at 100°C in the presence of 0.5 mM DTT. Labeling with mBBr was then carried out as described above except that the standards were heated for 2 min. at 100°C after the reaction was stopped with SDS and excess mBBr derivatized with  $\beta$ -mercaptoethanol. Because the intensity of the fluorescent bands was proportional to the amounts of added protein, it was assumed that reduction was complete under the conditions used.

#### EXAMPLE 1

##### Thioredoxin-linked Reduction of $\alpha$ -Amylase Inhibitors

##### Enzyme Activation Assays

The capability to replace a specific thioredoxin in the activation of chloroplast enzymes is one test for the ability of thiol groups of a given protein to undergo reversible redox change. Even though not physiological in the case of extra-plastidic proteins, this test has proved useful in several studies. A case in point is purothionin which, when reduced by thioredoxin *h* activates chloroplast FBPase (Wada, K. *et al.* (1981), *FEBS Lett.* 124:237-240, and Johnson, T.C., *et al.* (1987), *Plant Physiol.*, 85:446-451). The FBPase, whose physiological activator is thioredoxin *f*, is unaffected by thioredoxin *h*. In this Example, the ability of cystine-rich proteins to activate FBPase as well as NADP-MDH was tested as set forth above. The  $\alpha$ -amylase inhibitors from durum wheat (DSG-1 and DSG-2) were found to be effective in enzyme activation; however, they differed from purothionin in showing a specificity for NADP-MDH rather than FBPase (Table I). The  $\alpha$ -amylase inhibitors were active only in the presence of reduced thioredoxin *h*, which itself did not significantly activate NADP-MDH under these conditions (Fig. 1). As shown in Fig. 1, DSG-1 and DSG-2 activated NADP-malate dehydrogenase in the presence of DTT-reduced thioredoxin *h* according to the reaction sequence (DTT  $\rightarrow$  Thioredoxin  $\rightarrow$  DSG  $\rightarrow$  NADP-MDH).

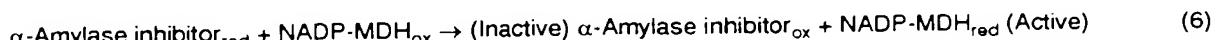
Fig. 1 represents results obtained with either the DSG-1 or DSG-2 inhibitors from durum wheat;  $\beta$ -MET refers to  $\beta$ -mercaptoethanol. The complete system for activation contained in 200  $\mu$ l of 100 mM Tris-HCl buffer, pH 7.9; 10 mM DTT; 0.7  $\mu$ g corn leaf NADP-MDH; 0.25  $\mu$ g wheat thioredoxin *h* and 10  $\mu$ g of DSG-1 or DSG-2. As indicated, 20 mM  $\beta$ -mercaptoethanol ( $\beta$ -MET) replaced DTT. Following preincubation, NADP-MDH was assayed spectrophotometrically.

In the enzyme activation assays, thioredoxin *h* was reduced by DTT; as expected, monothiols such as  $\beta$ -mercaptoethanol ( $\beta$ -MET), which do not reduce thioredoxin at a significant rate under these conditions (Jacquot, J.-P., *et al.* (1981), *Plant Physiol.* 68:300-304; Nishizawa, A.N., *et al.* (1982), "Methods in Chloroplast Molecular Biology", (M. Edelman, R.B. Hallick and N.-H. Chua, eds.) pp. 707-714, Elsevier Biomedical Press, New York, and Crawford, N.A., *et al.* (1989), *Arch. Biochem. Biophys.* 271:223-239), did not replace DTT.

NADP-MDH activity was proportional to the level of added DSG-1 and DSG-2 at a constant thioredoxin *h* concentration (see Fig. 2). Fig. 2 shows the effect of  $\alpha$ -amylase inhibitor concentration on NADP-malate dehydrogenase activation by DSG-1 and DSG-2 according to the same DTT formula set forth above. Except for varying the DSG-1 or DSG-2 concentrations, conditions were identical to those previously described and shown in Fig. 1. When tested at a fixed DSG concentration, NADP-MDH showed enhanced activity with increasing thioredoxin *h* (as shown in Fig. 3). Except for varying the thioredoxin *h* concentration, conditions were as described above for Fig. 1.

CM-1--the bread wheat protein that is similar to DSG proteins but has a lower molecular weight - also activated NADP-MDH and not FBPase when 20  $\mu$ g of CM-1 were used as shown in Table I. The results indicate that thioredoxin *h* reduces a variety of  $\alpha$ -amylase inhibitors, which, in turn, activate NADP-MDH in accordance with equations 4-6. These proteins were ineffective in enzyme activation when DTT was added in the absence of thioredoxin.





5

TABLE I

Effectiveness of Thioredoxin-Reduced Trypsin Inhibitors, Thionins, and  $\alpha$ -Amylase Inhibitors in Activating Chloroplast NADP-Malate Dehydrogenase and Fructose Bisphosphatase (DTT  $\rightarrow$  Thioredoxin  $\rightarrow$  Indicated Protein  $\rightarrow$  Target Enzyme)

Protein	M, kDa	No. of S-S Groups	*ACTIVITY, nkat/mg	
			NADP-MDH	FBPase
$\alpha$ -Amylase Inhibitors				
**DSG-2	17	5	2	0
**DSG-1	14	5	2	0
$\pm$ CM-1	12	5	12	0
Trypsin Inhibitors				
Cystine-rich (plant)				
Corn kernel	12	5	5	0
Soybean Bowman-Birk	8	7	3	0
Other types				
Ovomucoid	28	9	2	0
Soybean Kunitz	20	2	2	0
Ovoinhibitor	49	14	1	0
Bovine lung (Aprotinin)	7	3	Trace	2
Thionins				
**Purothionin- $\alpha_1$	6	4	1	39
**Purothionin- $\beta$	6	4	Trace	5
$\pm$ Purothionin- $\alpha$	6	4	0	14

\* These values compare to the corresponding values of 40 and 550 obtained, respectively, with spinach chloroplast thioredoxin  $\text{m}$  (NADP-MDH) and thioredoxin  $\text{f}$ .

\*\* From Durum wheat

$\pm$  From bread wheat

50

## EXAMPLE 2

### DTNB Reduction Assays

55 A second test for thiol redox activity is the ability to catalyze the reduction of the sulphydryl reagent, 2',5'-dithiobis(2-nitrobenzoic acid) (DTNB), measured by an increase in absorbance at 412 nm. Here, the protein assayed was reduced with NADPH via NTR and a thioredoxin. The DTNB assay proved to be effective for the  $\alpha$ -amylase inhibitors from both durum (DSG-1 and 2) and bread wheat (CM-1). When reduced by the NADP/thioredoxin system (in this case using

NTR and thioredoxin from *E. coli*), either DSG-1 or DSG-2 markedly enhanced the reduction of DTNB (Fig. 4). The uppermost curve in Fig. 4 represents results obtained with either DSG-1 or -2 (NADPH → NTR → Thioredoxin → DSG → DTNB). The DTNB reduction assay was carried out with 10 µg thioredoxin and 10 µg NTR and 20 µg of DSG-1 or DSG-2. CM-1 was also effective in the DTNB reduction assay, and, as with NADP-MDH activation (Table I), was detectably more active than the DSG proteins (See, Fig. 5, conditions were as in Fig. 4 except that the DSG proteins were omitted and purothionin  $\alpha$ , 20 µg or CM-1, 20 µg was used). The results thus confirmed the enzyme activation experiments in Example 1 and showed that the  $\alpha$ -amylase inhibitors can be reduced physiologically by the NADP/thioredoxin system. The role of the  $\alpha$ -amylase inhibitors in promoting the reduction of DTNB under these conditions is summarized in equations 7-9.



### EXAMPLE 3

#### Protein Reduction Measurements

20 The availability of monobromobimane (mBBr) and its adaptation for use in plant systems has given a new technique for measuring the sulphydryl groups of plant proteins (Crawford, N.A., et al. (1989), *Arch. Biochem. Biophys.* **271**:223-239). When coupled with SDS-polyacrylamide gel electrophoresis, mBBr can be used to quantitate the change in the sulphydryl status of redox active proteins, even in complex mixtures. This technique was therefore applied to the inhibitor 25 proteins to confirm their capacity for reduction by thioredoxin. Here, the test protein was reduced with thioredoxin which itself had been previously reduced with either DTT or NADPH and NTR. The mBBr derivative of the reduced protein was then prepared, separated from other components by SDS-polyacrylamide gel electrophoresis and its reduction state was examined by fluorescence. In the experiments described below, thioredoxin from *E. coli* was found to be effective in the reduction of each of the targeted proteins. Parallel experiments revealed that thioredoxin *h* and calf thymus thioredoxins reduced, respectively, the proteins from seed and animal sources.

30 In confirmation of the enzyme activation and dye reduction experiments, DSG-1 was effectively reduced in the presence of thioredoxin. Following incubation the proteins were derivatized with mBBr and fluorescence visualized after SDS-polyacrylamide gel electrophoresis (Fig. 6). Reduction was much less with DTT alone and was insignificant with GSH. A similar requirement for thioredoxin was observed for the reduction of CM-1 (Fig. 7) and DSG-2 (data not shown). While the thioredoxin used in Figs. 6 and 7 was from *E. coli* similar results were obtained with wheat thioredoxin *h*. Thioredoxin was also required when DTT was replaced by NADPH and NTR (data not shown).

### EXAMPLE 4

#### Thioredoxin-linked Reduction of Cystine-Rich Plant Trypsin Inhibitors

40 Whereas the major soluble cystine-rich proteins of wheat seeds can act as inhibitors of exogenous  $\alpha$ -amylases, the cystine-rich proteins of most other seeds lack this activity, and, in certain cases, act as specific inhibitors of trypsin from animal sources. While these proteins can be reduced with strong chemical reductants such as sodium borohydride (Birk, Y. (1985), *Int. J. Peptide Protein Res.* **25**:113-131, and Birl, Y. (1976), *Meth. Enzymol.* **45**:695-7390), there is little 45 evidence that they can be reduced under physiological conditions. It was therefore of interest to test trypsin inhibitors for the capacity to be reduced by thioredoxin. The cystine-rich representatives tested included the soybean Bowman-Birk and corn kernel trypsin inhibitors. The results in both cases were positive: each inhibitor activated NADP-MDH (but not FBPase) when added in the presence of DTT-reduced thioredoxin (Table I) and each reduced DTNB in the presence 50 of NADPH, NTR and thioredoxin (data not shown).

55 As found for the  $\alpha$ -amylase inhibitors, the thioredoxin-dependent reduction of the cystine-rich trypsin inhibitors could be directly monitored by the mBBr/SDS-polyacrylamide gel electrophoresis technique. Thus, significant reduction by DTT was observed only in the presence of reduced thioredoxin with both the Bowman-Birk (BBTI) inhibitor (highly fluorescent fast moving band in Fig. 6) and corn kernel (CKTI) trypsin inhibitor (highly fluorescent band migrating behind thioredoxin in Fig. 7).

EXAMPLE 5Thioredoxin-linked Reduction of Other Trypsin Inhibitors and Purothionins

5 In view of the finding that cystine-rich trypsin inhibitors from seeds can undergo specific reduction by thioredoxin, the question arose as to whether other types of trypsin inhibitor proteins share this property. In the course of this study, several such inhibitors --soybean Kunitz, bovine lung aprotinin, egg white ovoinhibitor and ovomucoid trypsin inhibitors -- were tested. While the parameters tested were not as extensive as with the cystine-rich proteins described above, it was found that the other trypsin inhibitors also showed a capacity to be reduced specifically by thioredoxin as measured 10 by both the enzyme activation and mBBr/SDS-polyacrylamide gel electrophoresis methods. As was the case for the cystine-rich proteins described above, the trypsin inhibitors tested in this phase of the study (soybean Kunitz and animal trypsin inhibitors) activated NADP-MDH but not FBPase (Table I). Bovine lung aprotinin was an exception in that it activated FBPase more effectively than NADP-MDH. It might also be noted that aprotinin resembles certain of the seed 15 proteins studied here in that it shows a high content of cystine (ca. 10%) (Kassel, B., et al. (1965), *Biochem. Biophys. Res. Commun.* 20:463-468).

The fluorescence evidence for the thioredoxin-linked reduction of one of these proteins, the Kunitz inhibitor, is shown in Fig. 7 (highly fluorescent slow moving band). In its reduced form, the Kunitz inhibitor also yielded a fluorescent fast moving band. The nature of this lower molecular mass species is not known. Its position suggests that it could represent Bowman-Birk inhibitor present as a contaminant in the Kunitz preparation (cf. Fig. 6); however, such a component was not evident in Coomassie Blue stained SDS gels. The animal inhibitors which yielded a single fluorescent 20 band of the expected molecular weight, also showed a thioredoxin requirement for reduction (data not shown).

In confirmation of earlier results, thioredoxin-reduced purothionin consistently activated FBPase and the type tested earlier, purothionin- $\alpha$ , failed to activate NADP-MDH (Table I) (Wada, K., et al. (1981), *FEBS Lett.* 124:237-240). However, in contrast to purothionin- $\alpha$  from bread wheat, two purothionins previously not examined (purothionins  $\alpha$ -1 25 and  $\beta$  from durum wheat) detectably activated NADP-MDH (Table I). The two durum wheat purothionins also differed in their ability to activate FBPase. The activity differences between these purothionins were unexpected in view of the strong similarity in their amino acid sequences (Jones, B.L., et al. (1977), *Cereal Chem.* 54:511-523) and in their ability to undergo reduction by thioredoxin. A requirement for thioredoxin was observed for the reduction of purothionin (here the  $\alpha$ -type) by the SDS-PAGE fluorescence procedure (Fig. 7).

EXAMPLE 6Quantitation of Reduction

35 The above Examples demonstrate that thioredoxin reduces a variety of proteins, including  $\alpha$ -amylase, such as the CM and DSG inhibitors, and trypsin inhibitors from seed as well as animal sources. While clear in the qualitative sense, the above results give no quantitative indication of the extent of reduction. Therefore, an experiment was conducted following the protocol of Crawford, et al. (Crawford, N.A., et al. (1989), *Arch. Biochem. Biophys.* 271:223-239).

40 As shown in Table II, the extent of reduction of the seed inhibitor proteins by the *E. coli* NADP/thioredoxin system was time-dependent and reached, depending on the protein, 15 to 48% reduction after two hours. The results, based on fluorescence emitted by the major protein component, indicate that thioredoxin acts catalytically in the reduction of the  $\alpha$ -amylase and trypsin inhibitors. The ratio of protein reduced after two hours to thioredoxin added was greater than one for both the most highly reduced protein (soybean Bowman-Birk trypsin inhibitor) and the least reduced protein (corn kernel trypsin inhibitor) -- i.e., respective ratios of 7 and 2 after a two-hour reduction period. It should be noted 45 that the values in Table II were obtained under standard assay conditions and no attempt was made to optimize reduction by modifying those conditions.

TABLE II

Extent of Reduction of Seed proteins by the NADP/Thioredoxin System Using the mBBr/SDS-Polyacrylamide Gel Electrophoresis Procedure		
The following concentrations of proteins were used (nmoles): thioredoxin, 0.08; NTR, 0.01; purothionin- $\beta$ , 1.7; DSG-1, 0.7; corn kernel trypsin inhibitor, 1.0; Bowman-Birk trypsin inhibitor, 1.3; and Kunitz trypsin inhibitor, 0.5. Except for the indicated time difference, other conditions were as in Fig. 6.		
Protein	20 min	% Reduction After 120 min

TABLE II (continued)

Extent of Reduction of Seed proteins by the NADP/Thioredoxin System Using the mBBr/SDS-Polyacrylamide Gel Electrophoresis Procedure		
The following concentrations of proteins were used (nmoles): thioredoxin, 0.08; NTR, 0.01; purothionin- $\beta$ , 1.7; DSG-1, 0.7; corn kernel trypsin inhibitor, 1.0; Bowman-Birk trypsin inhibitor, 1.3; and Kunitz trypsin inhibitor, 0.5. Except for the indicated time difference, other conditions were as in Fig. 6.		
		% Reduction After
Purothionin- $\beta$	15	32
DSG-1	22	38
Corn kernel trypsin inhibitor	3	15
Bowman-Birk trypsin inhibitor	25	48
Kunitz trypsin inhibitor	14	22

EXAMPLE 720 E. coli Glutaredoxin as Reductant

Bacteria and animals are known to contain a thiol redox protein, glutaredoxin, that can replace thioredoxin in reactions such as ribonucleotide reduction (Holmgren, A. (1985), *Annu. Rev. Biochem.* 54:237-271). Glutaredoxin is reduced as shown in equations 10 and 11.



0 So far there is no evidence that glutaredoxin interacts with proteins from higher plants. This ability was tested, using 5 glutaredoxin from *E. coli* and the seed proteins currently under study. Reduction activity was monitored by the under the conditions developed for Figs. 6 and 7, glutaredoxin could effectively replace thioredoxin in some, but not all cases. Thus, glutaredoxin was found to be active in the reduction of the following (the numbers indicate the percentage reduction relative to *E. coli* thioredoxin): DSG-1 and CM-1  $\alpha$ -amylase inhibitors (147 and 210%, respectively); corn kernel trypsin inhibitor (424%); and purothionin (82, 133, and 120% for the  $\alpha$ ,  $\alpha$ 1 and  $\beta$  forms, respectively). Glutaredoxin was ineffective in the reduction of the DSG-2  $\alpha$ -amylase inhibitor and the soybean Bowman-Birk and Kunits trypsin inhibitors. The trypsin inhibitors from animal sources also showed a mixed response to glutaredoxin. Egg white ovoinsulin and bovine lung aprotinin were not affected. Significantly, as previously reported (Wolosiuk, R.A., et al. (1977), *Nature* 266:565-567), glutaredoxin failed to replace thioredoxin as the immediate reductant in the activation of thioredoxin-linked enzymes of chloroplasts, FBPase and NADP-MDH (data not shown).

5 The above Examples demonstrate that some of the enzyme inhibitor proteins tested can be reduced by glutaredoxin as well as thioredoxin. Those specific for thioredoxin include an  $\alpha$ -amylase inhibitor (DSG-2), and several trypsin inhibitors (Kunitz, Bowman-Birk, aprotinin, and ovomucoid inhibitor). Those proteins that were reduced by either thioredoxin or glutaredoxin include the purothionins, two  $\alpha$ -amylase inhibitors (DSG-1, CM-1), a cystine-rich trypsin inhibitor from plants (corn kernel inhibitor), and a trypsin inhibitor from animals (egg white ovoinsulin). These results raise the question of whether glutaredoxin occurs in plants. Glutaredoxin was reported to be present in a green alga (Tsang, M.L.-S. (1981), *Plant Physiol.* 68:1098-1104) but not in higher plants.

Although the activities of the NADP-MDH and FBPase target enzymes shown in Table I are low relative to those seen following activation by the physiological chloroplast proteins (thioredoxin  $\text{m}$  or  $\text{f}$ ), the values shown were found repeatedly and therefore are considered to be real. It seems possible that the enzyme specificity shown by the inhibitor proteins, although not relevant physiologically, reflects a particular structure achieved on reduction. It remains to be seen whether such a reduced structure is related to function within the seed or animal cell.

The physiological consequence of the thioredoxin (or glutaredoxin) linked reduction event is of considerable interest as the function of the targeted proteins is unclear. The present results offer a new possibility. The finding that thioredoxin reduces a wide variety of inhibitor proteins under physiological conditions suggests that, in the absence of compartmental barriers, reduction can take place within the cell.

EXAMPLE 8Inactivation of Soybean Trypsin Inhibitor in Soybean Meal

5 The goal of this Example is to inactivate the Bowman-Birk and Kunitz trypsin inhibitors of soybeans. The following protocol applies to animal feed preparations.

To 10 g of soybean meal are added 0.2 µg thioredoxin, 0.1 µg NADP-thioredoxin reductase and 500 nanomoles NADPH together with 1 M Tris-HCl buffer, pH 7.9, to give 5.25 ml of 30 mM Tris-HCl. The above mixture is allowed to sit for about 30 min. at room temperature. Direct reduction of the soybean trypsin inhibitor is determined using the mBBr 10 fluorescent labeling/SDS-polyacrylamide gel electrophoresis method previously described (Kobrehel, K., et al. (1991), *J. Biol. Chem.* **266**:16135-16140). An analysis of the ability of the treated flour for trypsin activity is made using modifications of the insulin and BAEE (Na-benzoyl-L-arginine ethyl ester) assays (Schoellmann, G., et al. (1963), *Biochemistry* **252**:1963; Gonias, S.L., et al. (1983), *J. Biol. Chem.* **258**:14682). From this analysis it is determined that soybean 15 meal so treated with the NADP/thioredoxin system does not inhibit trypsin.

EXAMPLE 9Inactivation of  $\alpha$ -Amylase Inhibitors in Cereals

20 To 10 g of barley malt are added 0.2 µg thioredoxin, 0.1 µg NADP-thioredoxin reductase and 500 nanomoles NADPH together with 1 M Tris-HCl buffer, pH 7.9, to give 5.25 ml of 30 mM Tris-HCl. The above mixture is allowed to sit for about 30 min. at room temperature. Direct reduction of the  $\alpha$ -amylase inhibitors is determined using the mBBr 25 fluorescent labeling/SDS-polyacrylamide gel electrophoresis method previously described (Kobrehel, K., et al. (1991), *J. Biol. Chem.* **266**:16135-16140).  $\alpha$ -Amylase activity is monitored by following the release of maltose from starch (Bernfeld, P. (1955), *Methods in Enzymol.* **1**:149). From this analysis it is determined that barley so treated with the NADP/thioredoxin system does not inhibit  $\alpha$ -amylase.

REDUCTION OF CEREAL PROTEINSMaterials and MethodsPlant Material

30 Seeds and semolina of durum wheat (*Triticum durum*, Desf. cv. Monroe) were kind gifts of Dr. K. Kahn.

Germination of wheat seeds

35 Twenty to 30 seeds were placed in a plastic Petri dish on three layers of Whatman #1 filter paper moistened with 5 ml of deionized water. Germination was carried out for up to 4 days at room temperature in a dark chamber.

Reagents/Fine Chemicals

40 Biochemicals and lyophilized coupling enzymes were obtained from Sigma Chemical Co. (St. Louis, MO). *E. coli* thioredoxin and NTR were purchased from American Diagnostica, Inc. (Greenwich, CT). Wheat thioredoxin *h* and NTR 45 were isolated from germ, following the procedures developed for spinach leaves (Florencio, F.J., et al. (1988), *Arch. Biochem. Biophys.* **266**:496-507). *E. coli* glutaredoxin was a kind gift of Professor A. Holmgren. Reagents for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). Monobromobimane (mBBr) or Thiolite was obtained from Calbiochem Co. (San Diego, CA). Aluminum lactate and methyl green were products of Fluka Chemicals Co. (Buchs, Switzerland).

Gliadins and Glutenins

50 For isolation of insoluble storage proteins, semolina (0.2 g) was extracted sequentially with 1 ml of the following 55 solutions for the indicated times at 25°C: (1) 50 mM Tris-HCl, pH 7.5 (20 min); (2) 70% ethanol (2 hr); and (3) 0.1 M acetic acid (2 hr). During extraction, samples were placed on an electrical rotator and, in addition, occasionally agitated with a vortex mixer. After extraction with each solvent, samples were centrifuged (12,000 rpm for 5 min.) in an Eppendorf microfuge and, supernatant fractions were saved for analysis. In between each extraction, pellets were washed with 1 ml of water, collected by centrifugation as before and the supernatant wash fractions were discarded. By conven-

tion, the fractions are designated: (1) albumin/globulin; (2) gliadin; and (3) glutenin.

In vitro mBBr labelling of proteins

5 Reactions were carried out in 100 mM Tris-HCl buffer, pH 7.9. As indicated, 0.7 µg NTR and 1 µg thioredoxin (both from *E. coli* unless specified otherwise) were added to 70 µl of this buffer containing 1 mM NADPH and 10 µg of target protein. When thioredoxin was reduced by dithiothreitol (DTT), NADPH and NTR were omitted and DTT was added to 10 0.5 mM. Assays with reduced glutathione were performed similarly, but at a final concentration of 1 mM. After incubation for 20 min, 100 nmoles of mBBr were added and the reaction was continued for another 15 min. To stop the reaction and derivatize excess mBBr, 10 µl of 10% SDS and 10 µl of 100 mM  $\beta$ -mercaptoethanol were added and the samples were then applied to the gels. For reduction by glutaredoxin, the thioredoxin and NTR were replaced by 1 µg *E. coli* glutaredoxin, 1.4 µg glutathione reductase (purified from spinach leaves) and 1.5 mM NADPH.

15 In vivo mBBr labelling of proteins

At the indicated times, the dry seeds or germinating seedlings (selected on the basis of similar radical length) were removed from the Petri dish and their embryos or germinated axes were removed. Five endosperms from each lot were weighed and then ground in liquid N<sub>2</sub> with a mortar and pestle. One ml of 2.0 mM mBBr in 100 mM Tris-HCl, pH 7.9, 20 buffer was added just as the last trace of liquid N<sub>2</sub> disappeared. The thawed mixture was then ground for another minute and transferred to a microfuge tube. The volume of the suspension was adjusted to 1 ml with the appropriate mBBr or buffer solution. Protein fractions of albumin/globulin, gliadin and glutenin were extracted from endosperm of germinated seedlings as described above. The extracted protein fractions were stored at -20°C until use. A buffer control was included for each time point.

25 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide electrophoresis of the mBBr-derivatized samples was performed in 15% gels at pH 8.5 as described by Laemmli, U.K. (1970), *Nature* 227:680-685. Gels of 1.5 mm thickness were developed for 16 hr. at a constant current of 9 mA.

30 Native Gel Electrophoresis

To resolve the different types of gliadins, native polyacrylamide gel electrophoresis was performed in 6% gels (a procedure designed to separate gliadins into the four types) as described by Bushuk and Zillman (Bushuk, W., et al. 35 (1978), *Can. J. Plant Sci.* 58:505-515) and modified for vertical slab gels by Sapirstein and Bushuk (Sapirstein, H.D., et al. (1985), *Cereal Chem.* 62:372-377). A gel solution in 100 ml final volume contained 6.0 g acrylamide, 0.3 g bisacrylamide, 0.024 g ascorbic acid, 0.2 mg ferrous sulfate heptahydrate and 0.25 g aluminum lactate. The pH was adjusted to 3.1 with lactic acid. The gel solution was degassed for 2 hr. on ice and then 0.5 ml of 3% hydrogen peroxide was 40 added as a polymerization catalyst. The running buffer, also adjusted to pH 3.1 with lactic acid, contained 0.5 g aluminum lactate per liter. Duration of electrophoresis was approximately 4 hr., with a constant current of 50 mA. Electrophoresis was terminated when the solvent front, marked with methyl green tracking dye, migrated to about 1 cm from the end of the gel.

45 mBBr Removal/Fluorescence Photography

Following electrophoresis, gels were placed in 12% (w/v) trichloroacetic acid and soaked for 4 to 6 hr. with one change of solution to fix the proteins; gels were then transferred to a solution of 40% methanol/10% acetic acid for 8 to 10 hr. to remove excess mBBr. The fluorescence of mBBr, both free and protein bound, was visualized by placing gels 50 on a light box fitted with an ultraviolet light source (365 nm). Following removal of the excess (free) mBBr, gels were photographed with Polaroid Positive/Negative Landfilm, type 55, through a yellow Wratten gelatin filter No. 8 (cutoff=460 nm) (exposure time ranged from 25 to 60 sec at f4.5) (Crawford, N.A., et al. (1989), *Arch. Biochem. Biophys.* 271:223-239).

55 Protein Staining/Destaining/Photography

SDS-gels were stained with Coomassie Brilliant Blue R-250 in 40% methanol/10% acetic acid for 1 to 2 hr. and destained overnight as described before (Crawford, N.A., et al. (1989), *Arch. Biochem. Biophys.* 271:223-239). Aluminum lactate native gels were stained overnight in a filtered solution containing 0.1 g Coomassie Brilliant Blue R-250

(dissolved in 10 ml 95% ethanol) in 240 ml 12% trichloroacetic acid. Gels were destained overnight in 12% trichloroacetic acid (Bushuk, W., *et al.* (1978), *Can. J. Plant Sci.* 58:505-515, and Sapirstein, H.D., *et al.* (1985), *Cereal Chem.* 62:372-377).

5 Protein stained gels were photographed with Polaroid type 55 film to produce prints and negatives. Prints were used to determine band migration distances and loading efficiency.

The Polaroid negatives of fluorescent gels and prints of wet protein stained gels were scanned with a laser densitometer (Pharmacia-LKB UltroScan XL). Fluorescence was quantified by evaluating peak areas after integration with GelScan XL software.

10 Enzyme Assays

The following activities were determined in crude extracts with previously described methods: hexokinase (Baldus, B., *et al.* (1981), *Phytochem.* 20:1811-1814), glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Schnarrenberger, C., *et al.* (1973), *Arch. Biochem. Biophys.* 154:438-448), glutathione reductase, NTR and 15 thioredoxin h (Florencio, F.J., *et al.* (1988), *Arch. Biochem. Biophys.* 266:496-507).

Protein Determination

20 Protein concentrations were determined by the Bradford method (Bradford, M. (1976) *Anal. Biochem.* 72:248-256), with Bio-Rad reagent and bovine serum albumin as a standard.

Subunit Molecular Weight Determination

The subunit molecular weight of gliadins and glutenins was estimated on SDS-PAGE gels by using two sets of 25 molecular weight standards (kDa). The first set consisted of BSA (66), ovalbumin (45), soybean trypsin inhibitor (20.1), myoglobin (17), cytochrome c (12.4) and aprotinin (6.5). The other set was the BioRad Prestained Low SDS-PAGE standards: phosphorylase b (110), BSA (84), ovalbumin (47), carbonic anhydrase (33), soybean trypsin inhibitor (24) and lysozyme (16).

30 EXAMPLE 10

Reduction of Gliadins

As a result of the pioneering contributions of Osborne and coworkers a century ago, seed proteins can be fractionated on the basis of their solubility in aqueous and organic solvents (20). In the case of wheat, preparations of 35 endosperm (flour or semolina) are historically sequentially extracted with four solutions to yield the indicated protein fraction: (i) water, albumins; (ii) salt water, globulins; (iii) ethanol/water, gliadins; and (iv) acetic acid/water, glutenins. A wide body of evidence has shown that different proteins are enriched in each fraction. For example, the albumin and globulin fractions contain numerous enzymes, and the gliadin and glutenin fractions are in the storage proteins required 40 for germination.

Examples 1, 4 and 5 above describe a number of water soluble seed proteins (albumins/globulins, e.g.,  $\alpha$ -amylase inhibitors, cystine-rich trypsin inhibitors, other trypsin inhibitors and thionines) that are reduced by the NADP/thioredoxin system, derived either from the seed itself or *E. coli*. The ability of the system to reduce insoluble storage proteins from wheat seeds, viz., representatives of the gliadin and glutenin fractions, is described below. Following incubation 45 with the indicated additions, the gliadin proteins were derivatized with mBBr and fluorescence was visualized after SDS-polyacrylamide gel electrophoresis. The lanes in Fig. 8 were as follows: 1. Control: no addition. 2. GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach leaves) and NADPH. 3. NGS: NADPH, reduced glutathione, glutathione reductase (from spinach leaves) and glutaredoxin (from *E. coli*). 4. NTS: NADPH, NTR, and thioredoxin (both proteins from *E. coli*). 5. MET/T(Ec):  $\beta$ -mercaptoethanol and thioredoxin (*E. coli*). 6. DTT. 7. DTT/T(Ec): DTT and 50 thioredoxin (*E. coli*). 8. DTT/T(W): Same as 7 except with wheat thioredoxin h. 9. NGS,-Gliadin: same as 3 except without the gliadin protein fraction. 10. NTS,-Gliadin: same as 4 except without the gliadin protein fraction. Based on its reactivity with mBBr, the gliadin fraction was extensively reduced by thioredoxin (Fig. 8). The major members undergoing reduction showed a Mr ranging from 25 to 45 kDa. As seen in Examples 1, 4 and 5 with the seed  $\alpha$ -amylase and trypsin inhibitor proteins, the gliadins were reduced by either native h or *E. coli* type thioredoxin (both homogeneous); 55 NADPH (and NTR) or DTT could serve as the reductant for thioredoxin. Much less extensive reduction was observed with glutathione and glutaredoxin--a protein able to replace thioredoxin in certain *E. coli* and mammalian enzyme systems, but not known to occur in higher plants.

The gliadin fraction is made up of four different protein types, designated  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\omega$ , which can be separated by

native polyacrylamide gel electrophoresis under acidic conditions (Bushuk, W., et al. (1978), *Can. J. Plant Sci.* 58:505-515; Kasarda, D.D., et al. (1976), *Adv. Cer. Sci. Tech.* 1:158-236; Sapirstein, H.D., et al. (1985), *Cereal Chem.* 62:372-377; and Tatham, A.S., et al. (1990), *Adv. Cer. Sci. Tech.* 10:1-78). Except for the  $\omega$  gliadins, each species contains cysteine (S-S) groups and thus has the potential for reduction by thioredoxin. In this study, following incubation with the indicated additions, proteins were derivatized with mBBr, and fluorescence was visualized after acidic-polyacrylamide gel electrophoresis. The lanes in Fig. 9 were as follows: 1. Control: no addition. 2. GSH: reduced glutathione. 3. GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach leaves) and NADPH. 4. NGS: NADPH, reduced glutathione, glutathione reductase (from spinach leaves) and glutaredoxin (from *E. coli*). 5. NGS+NTS: combination of 4 and 6. 6. NTS: NADPH, NTR, and thioredoxin (both proteins from *E. coli*). 7. MET/T(Ec):  $\beta$ -mercaptoethanol and thioredoxin (*E. coli*). 8. DTT/T(Ec): DTT and thioredoxin (*E. coli*). 9. NTS(-T): same as 6 except without thioredoxin. 10. NGS+NTS,-Gliadin: same as 5 except without the gliadin fraction.

When the thioredoxin-reduced gliadin fraction was subjected to native gel electrophoresis, the proteins found to be most specifically reduced by thioredoxin were recovered in the  $\alpha$  fraction (See, Fig. 9). There was active reduction of the  $\beta$  and  $\gamma$  gliadins, but as evident from the densitometer results summarized in Table III, the reduction within these groups was nonspecific, i.e., relatively high levels of reduction were also achieved with glutathione and glutaredoxin. There was especially strong reduction of the  $\gamma$  gliadins by DTT-reduced thioredoxin (Fig. 9). As anticipated, there was no reduction of the  $\omega$  gliadins. The evidence indicates that thioredoxin (either native *h* or *E. coli*) specifically reduces certain of the gliadins, especially the  $\alpha$  type.

20

TABLE III

Reductant Specificity of the Different Types of Gliadins				
Reductant	Gliadin, % Relative Reduction			
	$\alpha$	$\beta$	$\gamma$	Aggregate*
None	22.4	30.4	24.3	29.2
Glutathione	36.4	68.1	60.6	60.1
Glutaredoxin	43.5	83.3	79.7	61.5
Thioredoxin	100.0	100.0	100.0	100.0

\*Proteins not entering the gel

#### EXAMPLE 11

40

#### Reduction of Glutenins

The remaining group of seed proteins to be tested for a response to thioredoxin -- the glutenins -- while the least water soluble, are perhaps of greatest interest. The glutenins have attracted attention over the years because of their importance for the cooking quality of flour and semolina (MacRitchie, F., et al. (1990), *Adv. Cer. Sci. Tech.* 10:79-145). Testing the capability of thioredoxin to reduce the proteins of this group was, therefore, a primary goal of the current investigation.

As seen in Fig. 10 (treatments were as in Example 10, Fig. 8), several glutenins were reduced specifically by thioredoxin. The most extensive reduction was observed in the low molecular mass range (30 - 55 kDa). The reduction observed in the higher molecular mass range was less pronounced, but still obvious--especially in the 100 kDa region and above. Though not shown reduction may also occur in the 130 kDa range. Like the gliadins, certain of the glutenins were appreciably reduced by glutathione and glutaredoxin. However, in all cases, reduction was greater with thioredoxin and, in some cases, specific to thioredoxin (Table IV, note proteins in the 30 - 40 and 60 - 110 kDa range). As observed with the other wheat proteins tested, both the native *h* and *E. coli* thioredoxins were active and could be reduced with either NADPH and the corresponding NTR or with DTT. Thus as found for the gliadins, certain glutenins were reduced *in vitro* specifically by thioredoxin, whereas others were also reduced, albeit less effectively, by glutathione and glutaredoxin.

TABLE IV

Reductant Specificity of Glutenins			
Reaction conditions as in Fig. 3.			
Reductant	Glutenin, % Relative Reduction*		
	60-110 kDa	40-60 kDa	30-40 kDa
None	8	23	16
Glutathione	31	51	29
Glutaredoxin	50	72	40
Thioredoxin*	100	100	100

\*Area under the three molecular weight classes (from high to low) following reduction by the NADP/thioredoxin system were: 1.5, 5.67 and 5.04 Absorbance units times millimeters, respectively.

### EXAMPLE 12

#### In vivo Reduction Experiments

The above Example demonstrates that thioredoxin specifically reduces components of the wheat gliadin and glutenin fractions when tested *in vitro*. The results, however, provide no indication as to whether these proteins are reduced *in vivo* during germination -- a question that, to our knowledge, had not been previously addressed (Shutov, A.D., *et al.* (1987), *Phytochem.* **26**:1557-1566).

To answer this question, we applied the mBBr/SDS-PAGE technique was applied to monitor the reduction status of proteins in the germinating seed. We observed that reduction of components in the Osborne fractions increased progressively with time and reached a peak after 2 to 3 days germination (Fig. 11). The observed increase in reduction ranged from 2-fold with the gliadins, to 3-fold with the albumin/globulins and 5-fold with the glutenins. The results suggest that, while representatives of the major wheat protein groups were reduced during germination, the net redox change was greatest with the glutenins.

Although providing new evidence that the seed storage proteins undergo reduction during germination, the results of Fig. 11 give no indication as to how reduction is accomplished, *i.e.*, by glutathione or thioredoxin. To gain information on this point, the *in vivo* reduction levels of the principal thioredoxin-linked gliadins (30 - 50 kDa) and glutenins (30 - 40, 40 - 60 kDa) was compared with the reduction determined from *in vitro* measurements (cf. Fig. 8 and Table IV). For this purpose, the ratio of fluorescence to Coomassie stained protein observed *in vivo* during germination and *in vitro* with the appropriate enzyme reduction system was calculated. The results shown in Fig. 12 (principal thioredoxin linked gliadins were those in the Mr range from 25 to 45 kDa, see Fig. 8, and glutenins were those in the Mr range from 30 to 60 kDa, see Fig. 10) suggest that, while glutathione could account for a significant part of the *in vivo* reduction of the gliadin fraction (up to 90%), this was not the case with the glutenins whose reduction seemed to require thioredoxin. The level of reduction that could be ascribed to glutathione (or glutaredoxin) was insufficient to account for the levels of reduced glutenin measured in the germinating seed.

### EXAMPLE 13

#### Enzyme Measurements

The source of NADPH needed for the NTR linked reduction of thioredoxin *h* was also investigated. Semolina was analyzed for enzymes that function in the generation of NADPH in other systems, notably dehydrogenases of the oxidative phosphate pathway. The results summarized in Table V confirm earlier evidence that endosperm extracts contain the enzymes needed to generate NADPH from glucose via this pathway: hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Tatham, A.S., *et al.* (1990), *Adv. Cer. Sci. Tech.* **10**:1-78). It is noteworthy that the glucose 6-phosphate dehydrogenase activity seen in Table V was insensitive to reduced thioredoxin (data not shown). In this respect the endosperm enzyme resembles its cytosolic rather than its chloroplast counterpart from leaves (Fickenscher, K., *et al.* (1986), *Arch. Biochem. Biophys.* **247**:393-402; Buchanan, B.B. (1991), *Arch. Biochem.*

Biophys. 288:1-9; Scheibe, R., et al. (1990), Arch. Biochem. Biophys. 274:290-297).

As anticipated from earlier results with flour (Johnson, T.C., et al. (1987), Planta 171:321-331; Suske, G., et al. (1979), Z. Naturforsch. C 34:214-221), semolina also contained thioredoxin h and NTR (Table V). Interestingly, based on activity measurements, NTR appeared to be a rate-limiting component in preparations from the cultivar examined.

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TABLE V

Activities of Enzymes Effecting the Reduction of Thioredoxin h in Semolina (Glucose→Glu-6-P→6-P-Gluconate→NADP→Thioredoxin h)	
Protein	Activity (nkat/mg protein)
Hexokinase	0.28
Glucose-6-P dehydrogenase	0.45
6-P-Gluconate dehydrogenase	0.39
NTR	0.06
Thioredoxin h	0.35

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The present results suggest that thioredoxin h functions as a signal to enhance metabolic processes associated with the germination of wheat seeds. Following its reduction by NTR and NADPH (generated via the oxidative pentose phosphate pathway), thioredoxin h appears to function not only in the activation of enzymes, but also in the mobilization

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of storage proteins.

#### EXAMPLE 14

##### Improvement of Dough Quality

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Dough quality was improved by reducing the flour proteins using the NADP/thioredoxin system. Reduced thioredoxin specifically breaks sulfur-sulfur bonds that cross-link different parts of a protein and stabilize its folded shape. When these cross-links are cut the protein can unfold and link up with other proteins in bread, creating an interlocking lattice that forms the elastic network of dough. The dough rises because the network traps carbon dioxide produced by yeast in the fermenting process. It is proposed that the reduced thioredoxin activated the gliadins and glutenins in flour letting them recombine in a way that strengthened the dough (Fig. 13). Reduced thioredoxin strengthened the protein network formed during dough making. For these tests, namely those shown in Fig. 14(c) and Fig. 15(d) (using 10 gm of either intermediate quality wheat flour obtained from a local miller in Montpellier, France (Fig. 14), or poor quality wheat also obtained from a local miller in Montpellier, France (Fig. 15), this poor quality wheat being mainly of the Apollo cultivar), 0.2 µg *E. coli* thioredoxin, 0.1 µg *E. coli* NADP-thioredoxin reductase and 500 nanomoles NADPH were added together with 1 M Tris-HCl, pH 7.9 buffer to give 5.25 ml of a 30 mM Tris-HCl enzyme system mixture. The reaction was carried out by mixing the enzyme system mixture with the 10 gm of the flour in a micro-farinograph at 30°C. As seen in Figs. 14 and 15, the resulting farinograph measurements showed a strengthening of the dough by the added NADP/thioredoxin system. With a flour of poor quality, as in Fig. 15(d), the farinograph reading was stable for at least 4 min. after the dough was formed in the presence of the reduction system, whereas the reading dropped immediately after dough formation in the control without this addition (see Fig. 15(a)). The improving effect was persistent and was maintained throughout the run. Expressed another way, the micro-farinograph reading is 375 Brabender units, 7 min. after dough formation with the poor quality wheat control (no added enzyme system) versus 450 Brabender units for the same poor quality wheat treated with components of the NADP/thioredoxin system (NADPH, thioredoxin and NADP-thioredoxin reductase).

Another farinograph study was carried out as above with 10 gm of Apollo flour only the concentration of NADPH was 500 µmoles instead of nanomoles. As shown in the farinograph measurements in Fig. 16 this amount of NADPH also resulted in a definite improvement in the quality of the dough.

Higher farinograph measurements of dough correspond to improved dough strength and improved baked good characteristics such as better crumb quality, improved texture and higher loaf volume. Also, based on *in vivo* analyses with the isolated proteins, the native wheat seed NADP/thioredoxin system will also be effective in strengthening the dough.

For purposes of baking and other aspects of this invention, ranges of about 0.1 to 3.0 µg of a thioredoxin (preferably

5 *E. coli* or thioredoxin h) and from about 0.1 to 2.0  $\mu$ g reductase and about 30 to 500 nanomoles of NADPH are added for about every 10 gm of flour. The optimal levels of thioredoxin and reductase depend on flour quality. In general, the higher the flour quality, the higher the level of thioredoxin and reductase required. Thioredoxin can also be reduced by lipoic acid instead of by the NADPH/NADP-thioredoxin reductase reduction system. The other dough ingredients such as milk or water are then added. However, the liquid may first be added to the NTR/thioredoxin system and then added to the flour. It is preferred that yeast for purposes of leavening be added after the reduced thioredoxin has had a chance to reduce the storage proteins. The dough is then treated as a regular dough proofed, shaped, etc. and baked.

10 NADPH can be replaced in this Example as well as in the following Examples with an NADPH generator such as one consisting of 100  $\mu$ M glucose 6-phosphate, 100  $\mu$ M NADP and 0.05 units (0.2  $\mu$ gram) glucose 6-phosphate dehydrogenase from a source such as yeast. The NADPH generator is added together with thioredoxin and NADP-thioredoxin reductase at the start of the dough making process.

15 Fig. 17(c) shows the higher farinograph measurement obtained when 10 gm of Apollo cultivar (CV) wheat are reacted with 20  $\mu$ l NADP (25 mM), 20  $\mu$ l G6P (25 mM), 0.25  $\mu$ g G6PDase, 0.1  $\mu$ g NTR and 0.2  $\mu$ g thioredoxin h contained in 4.25 ml  $H_2O$  and 0.90 ml Tris-HCl (30 mM, pH 7.9). Fig. 17(b) shows that a higher farinograph measurement is also obtained when 10 gm of Apollo wheat are reacted with the same reaction mixture as the mixture resulting in Fig. 17(c) but without any NTR or thioredoxin.

#### EXAMPLE 15

##### 20 Wheat Bread Baking Studies

The baking tests were carried out by using a computer monitored PANASONIC baking apparatus.

25 Composition of bread:

Control:

30	Flour*:	200 gm (dry)
	Water:	70% hydratation
35	Salt (NaCl):	5.3 g
	Yeast:	4.8 g ( <i>Saccharomyces cerevisiae</i> , SafInstant) (dry yeast powder)

\* Flour samples were obtained from pure bread wheat cultivars having contrasting baking quality (including animal feed grade and other grades having from poor to good baking quality).

##### 40 Assays:

The dough for the assays contained all the components of the control plus as indicated varying amounts of the NADP Thioredoxin System (NTS) and/or the NADP generating System.

##### 45 Experimental conditions

- Flour and salt are weighed and mixed
- The volume of water needed to reach a hydration of 70% was put into the baking pan.
- 50 -- The mixture of flour and salt was added to the water and the baking program monitored by the computer was started. The complete program lasted 3 hrs 9 min and 7 secs.
- In the case of the assays, enzyme system components are added to the water before the addition of the flour-salt mixture.
- Yeast was added automatically after mixing for 20 min and 3 secs.

55 The program monitoring the Panasonic apparatus was:

Segments	Duration	Mixing Conditions	Heating
Mixing	00:00:03	T1	off
Mixing	00:05:00	T2	off
Mixing	00:05:00	T1	off
Rest	00:10:00	TO	off
Mixing	00:17:00	T2	off
Mixing	00:07:00	T1	off
Rest	00:30:00	TO	to reach 32°C
Mixing	00:00:04	T1	32°C
Rest	01:15:00	TO	32°C
Baking	00:14:00	TO	to reach 180°C
Baking	00:26:00	TO	180°C
Mixing Conditions:			
TO = no mixing (motor at rest)			
T1 = normal mixing			
T2 = alternately 3 second mixing, 3 second rest			

Bread loaf volume was determined at the end of the baking, when bread loaves reached room temperature.

Cultivar THESEE Assay

The french wheat cultivar Thesee is classified as having good breadmaking quality. Table VI below sets forth the results of the assay.

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TABLE VI

	NADPH ( $\mu$ moles)	NTR ( $\mu$ g)	Th ( $\mu$ g)	Loaf Volume	
				(cm <sup>3</sup> )	Relative Units
5	Control	0	0	1690	100
10	Samples	6.0	30	1810	107
		6.0	30	1725	102
		6.0	0	1720	102
		6.0	0	1550	92
		0	30	1800	107

15 \*NADPH  
Generating syst. 30 60 1620 96

20 \*NADPH  
Generating  
syst. plus  
ATP, glucose 30 60 1630 96

25 NTR and  
Th from  
yeast 6.0 9.4 20 1750 104

30 35 \*Composition of the NADPH generating system, ATP and  
glucose.

	Volume Added
NADP, 25 mMolar	700 $\mu$ l (17.5 $\mu$ moles)
Glucose-6-phosphate, 25 mMolar	700 $\mu$ l (17.5 $\mu$ moles)
Glucose-6-phosphate dehydrogenase (50 $\mu$ g/ml)	175 $\mu$ l (8.75 $\mu$ g)
ATP, 25 mMolar	700 $\mu$ l (17.5 $\mu$ moles)
Glucose, 25 mMolar	700 $\mu$ l (17.5 $\mu$ moles)

40 45 As shown in Table VI, an increased loaf volume was obtained when the complete NTS at concentrations of 6.0  $\mu$ moles NADPH, 30  $\mu$ g NTR and 60  $\mu$ g Th was used to bake loaves from 200 g of Thesee flour with the amounts and conditions described above in this Example. Unless otherwise stated, the NTR and thioredoxin (th) were from *E. coli*. No similar increase occurred when the generating system was used or when either NTR or Th were omitted. Also no significant effect on loaf volume occurred when amounts of the components in the system were about half or less than half of the amounts of above.

55 Cultivar APOLLO Assay

This French wheat cultivar is classified as having poor breadmaking quality. The NTR and thioredoxin used in this assay were from *E. coli*. Table VII below sets forth the results of this assay using 200 gm of Apollo flour. Again unless

otherwise stated the amounts and conditions are those described above at the beginning of the Example.

TABLE VII

				Loaf Volume	
	NADPH ( $\mu$ moles)	NTR ( $\mu$ g)	Th ( $\mu$ g)	(cm <sup>3</sup> )	Relative Units
Control	0	0	0	1400	100
Samples	6.0	30	60	1475	105
*NADPH Generating syst. plus ATP, glucose		30	60	1530	109
*NADPH Generating syst. plus ATP, glucose		0	0	1430	102
*NADPH Generating syst.		6	0	1430	102
*NADPH Generating syst.		6	7	1440	103

\*The composition of the generating system, ATP and glucose is as in Table VI.

20 Cultivar ARBON Assay

25 The French wheat cultivar Arbon is used for feed and is classified as non suitable for breadmaking. Tables VIII and IX below show that an improved bread loaf volume can be obtained from Arbon using the NTS or NADPH and NTR with the dough components and conditions described at the beginning of the Example. The amounts of NTR, thioredoxin, NADPH and the NADPH generating system components used in the assay are set forth in Tables VIII and IX. The improvement in Arbon bread quality using the complete NTS as set forth in Table IX is also clearly seen in the photographs shown in Figs. 18-22 and 23(a).

TABLE VIII

	NADPH ( $\mu$ moles)	NTR ( $\mu$ g)	Th ( $\mu$ g)	Loaf Volume (cm <sup>3</sup> )
Control	0	0	0	1350
Samples	0.1-0.6 >2.0	3-4 >20	3-4 >20	up to 20% higher than the control less than the control

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TABLE IX

		Loaf Volume	
		(cm3)	Relative Units
Treatment			
5	Complete NTS	1650	122
10	minus Thioredoxin	1690	125
15	minus NTR	1520	113
20	minus Thioredoxin, NTR	1540	114
25	minus NADPH	1440	107
	minus NADPH, plus *NADPH generating system	1560	116
	minus NTS (control)	1350	100
	NADPH, 0.6 $\mu$ moles		
	Thioredoxin, 3.5 $\mu$ g		
	NTR, 3 $\mu$ g		

\*Generating System:

3.5  $\mu$ moles NADP

3.5  $\mu$ moles glucose-6-phosphate

1.75  $\mu$ g glucose-6-phosphate dehydrogenase

#### EXAMPLE 16

##### Triticale Bread Baking Study

Triticale is a wheat/rye hybrid and is generally used for chicken feed. It is more nutritious than wheat but is not generally considered appropriate for breadmaking, especially in the more developed nations. The effect of the NTS system and variations thereof on loaves baked from Triticale flour was consequently studied. Unless otherwise stated, the baking conditions and dough ingredient were as described for wheat flour in Example 15. As shown in Table X there is an improvement in loaf volume when the triticale dough contained thioredoxin, NTR and the NADPH generating system in the amounts set forth in that Table. However, no corresponding improvement was seen when the NTS (i.e., thioredoxin, NTR and NADPH) was used. Fig. 24 shows that an improvement in the texture of the bread also occurred when NTR, Th and the NADPH generating system as set forth in Table X were used. The loaf on the right in Fig. 24 is the control.

TABLE X

Effect of the NADP/Thioredoxin System (NTS) on Loaves Baked from Triticale Flour (cv. Juan)		
Loaf Volume		
Treatment	(cm3)	Relative Units
Complete NTS	1230	94
minus NTS (control)	1310	100

TABLE X (continued)

Effect of the NADP/Thioredoxin System (NTS) on Loaves Baked from Triticale Flour (cv. Juan)		
Treatment	Loaf Volume	
	(cm <sup>3</sup> )	Relative Units
minus NADPH, plus *NADPH generating system	1390	106
NADPH, 0.6 $\mu$ moles Thioredoxin, 3.5 $\mu$ g NTR, 3.0 $\mu$ g Generating System: 4.5 $\mu$ moles NADP 4.5 $\mu$ moles glucose-6-phosphate 4.5 $\mu$ g glucose-6-phosphate dehydrogenase		

EXAMPLE 17

The effect of the NADPH/thioredoxin system on flour from sorghum, corn and rice was also determined. The baking conditions were as described for wheat flour in Example 15. The amounts of the components of the NTS as used in this assay were as follows: 8  $\mu$ moles NADPH, 40.5  $\mu$ g NTR and 54  $\mu$ g thioredoxin. Both the thioredoxin and NTR were from *E. coli*. The results of this assay are shown in Fig. 25 and also in Fig. 23(b). As shown in these figures the breads containing the NTS, especially corn and sorghum exhibited improved texture and stability.

EXAMPLE 18Reduction of Ethanol-Soluble and Myristate-Soluble Storage Proteins from Triticale, Rye, Barley, Oat, Rice, Sorghum, Corn and Teff

Unless otherwise stated, the materials and methods used in this Example are according to those set forth above in the section titled "Reduction of Cereal Proteins, Materials and Methods."

Triticale, Rye, Barley, Oat and Teff

The reactions were carried out in 30 mM Tris-HCl buffer, pH 7.9. As indicated, 0.7  $\mu$ g of NTR and 1  $\mu$ g of thioredoxin from *E. coli* or 2  $\mu$ g of thioredoxin from yeast, as identified, were added to 70  $\mu$ L of this buffer containing 1 mM NADPH and 25 to 30  $\mu$ g of extracted storage protein. The ethanol extracted storage proteins were obtained by using 50 ml of 70% ethanol for every 10 gm of flour and extracting for 2 hr. In the case of teff, 200 mg of ground seeds were extracted. The myristate extracted proteins were obtained by extracting 1 gm of flour with 8 mg sodium myristate in 5 ml of distilled H<sub>2</sub>O for 2 hrs. The combination of NADPH, NTR and thioredoxin is known as the NADP/thioredoxin system (NTS). As indicated, glutathione (GSH), 2.5 mM, was added as reductant in either the absence (GSH) or presence of 1.5 mM NADPH and 1.4  $\mu$ g of spinach leaf glutathione reductase (GR/GSH/NADPH). After incubation for 20 min, 100 nmol of mBBr was added and the reaction was continued for another 15 min. To stop the reaction and derivatize excess mBBr, 10  $\mu$ L of 10% SDS and 10  $\mu$ L of 100 mM 2-mercaptoethanol were added, and the samples were then applied to the gels. The procedure for SDS-polyacrylamide gel electrophoresis was as described by N. A. Crawford, *et al.* (1989 Arch. Biochem. Biophys. 271:223-239).

Rice, Sorghum and Corn

The reactions were carried out in 30 mM Tris-HCl buffer, pH 7.9. When proteins were reduced by thioredoxin, the following were added to 70  $\mu$ L of buffer: 1.2 mM NADPH, 10 to 30  $\mu$ g of seed protein fraction, 0.5  $\mu$ g *E. coli* NTR and 1  $\mu$ g *E. coli* thioredoxin. For reduction with glutathione, thioredoxin and NTR were replaced with 2.5 mM reduced glutathione and 1  $\mu$ g glutathione reductase (baker's yeast, Sigma Chemical Co.). For reduction with dithiothreitol, NADPH, thioredoxin, and NTR were omitted and 0.5 mM dithiothreitol was added. In all cases, incubation time was 20 min. Then 10  $\mu$ L of a 10 mM mBBr solution was added and the reaction continued for an additional 15 min. To stop the reaction and derivatize excess mBBr, 10  $\mu$ L of 10% SDS and 10  $\mu$ L of 100 mM 2-mercaptoethanol were added and the samples applied to the gels. In each case, to obtain the extracted protein, 1 g ground seeds was extracted with 8 mg of sodium

myristate in 5 ml distilled water. With the exception of the initial redox state determination of the proteins, samples were extracted for 2 hr at 22°C and then centrifuged 20 min at 16,000 rpm prior to the addition of the mBBr. With the initial redox state determination, the mBBr was added under a nitrogen atmosphere along with the myristate followed by extraction.

5 Figs. 26-30 represent pictures of the gels for the reduction studies of myristate-extracted proteins from flour of oat, triticale, rye, barley and teff. Buffer and ethanol-extracted proteins are also shown for teff in Fig. 30. In all of the studies represented by Figs. 26-30, the flour was first extracted with buffer, 50 mM Tris-HCl, pH 7.5 for 20 min. and then with 70% ethanol for 2 hr. Also shown are pictures of the gels for the myristate-extracted proteins from corn, sorghum and rice (Figs. 31 and 32). With corn, sorghum and rice, the ground seeds were extracted only with myristate. Therefore, 10 with corn, sorghum and rice, the myristate extract represents total protein, whereas with oat, triticale, rye, barley and teff, the myristate extract represents only the glutenin-equivalent fractions since these flours had been previously extracted with buffer and ethanol. The results, depicted in the gels in Figs. 26-30, show that the NTS is most effective, as compared to GSH or GSH/GR/NADPH, with myristate-extracted (glutenin-equivalent) proteins from oat, triticale, rye, 15 barley and teff. The NTS is also most effective with the total proteins from rice (Figs. 31 and 32). Reduced glutathione is more effective with the total proteins from corn and sorghum (Figs. 31 and 32).

#### Conclusions from Figs. 31 and 32 (corn, sorghum and rice)

20 As depicted in Fig. 31 in treatment (1), extraction with myristate in the presence of mBBr was carried out under a nitrogen atmosphere; in treatment (2), to the myristate extracted proteins mBBr was added without prior reduction of the proteins; in treatment (3), the myristate extracted proteins were reduced by the NADP/thioredoxin system (NTS); in treatment (4) the myristate extracted proteins were reduced by NADPH, glutathione and glutathione reductase. As depicted in Fig. 32, treatment (1) is like treatment (2) in Fig. 31; in treatment (2) the seeds were extracted with myristate in the presence of mBBr under nitrogen; in treatment (3), seeds were extracted with myristate and reduced by the NTS 25 and then mBBr was added; and in treatment (4) conditions as in (3) except that proteins were reduced by DTT. Treatment (1) in Fig. 31 and treatment (2) in Fig. 32 show the initial redox state of the proteins in the grains. For all three cereals, the proteins in the seed are highly reduced. If extracted in air, the proteins become oxidized especially the sorghum and rice. The oxidized proteins can be re-reduced, maximally with NTS in all cases. With rice, the reduction is relatively specific for thioredoxin; with corn, glutathione is as effective as thioredoxin and with sorghum glutathione is 30 slightly more effective than thioredoxin. Dithiothreitol showed varying effectiveness as a reductant. These experiments demonstrate that the storage proteins of these cereals are less specific than in the case of wheat and suggest that thioredoxin should be tested both in the presence and absence of glutathione when attempting to construct a dough network.

35 Figs. 33 and 34 represent pictures of the gels resulting from the reduction studies of wheat glutenins and gliadins, respectively, by a yeast NADP/thioredoxin system. The glutenins were obtained by using 50 ml of 0.1 M acetic acid for every 10 gm of flour and extracting for 2 hr. The gliadins were obtained by using 50 ml of 70% ethanol for every 10 gm of flour and extracting for 2 hr. The experiment shows that the yeast system is highly active in reducing the two major groups of wheat storage proteins.

40 Figs. 35-38 represent pictures of gels for the reduction of ethanol-extracted proteins from flour of triticale, rye, oat and barley, respectively. The results show that the NTS is most effective with the ethanol-extracted proteins from triticale, rye and oat. The ethanol-extracted barley proteins are reduced in the control and thioredoxin or glutathione has little effect.

#### EXAMPLE 19

45 Effect of Thioredoxin-linked Reduction on the Activity and Stability of the Kunitz and Bowman-Birk Soybean Trypsin Inhibitor Proteins

#### Materials and Methods

##### Plant materials

50 Durum wheat (*Triticum durum*, Desf. cv. Monroe) was a kind gift of Dr. K. Kahn. Wheat germ was obtained from Sigma Chemical Co. (St. Louis, MO).

##### Chemicals and Enzymes

55 Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad

5 Laboratories (Richmond, CA), and DTT was from Boehringer Mannheim Biochemicals (Indianapolis, IN). L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (type XIII, T8640), subtilisin (type VIII: bacterial subtilisin Carlsberg, P538O), KTI (T9003), BBTI (T9777), azocasein, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). *E. coli* thioredoxin and NTR were isolated from cells transformed to overexpress each 10 protein. The thioredoxin strain containing the recombinant plasmid, pFPI, was kindly provided by Dr. J.-P. Jacquot (de La Motte-Guery *et al.*, 1991). The NTR strain containing the recombinant plasmid, pPMR21, was kindly provided by Drs. Marjorie Russel and Peter Model (Russel and Model, 1988). The isolation procedures used for these proteins were as described in those studies with the following changes: cells were broken in a Ribi cell fractionator at 25,000 psi and NTR was purified as described by Florencio *et al.* (1988) without the red agarose step. The *E. coli* thioredoxin and NTR were, respectively, 100% and 90% pure as determined by SDS-polyacrylamide gel electrophoresis. Wheat thioredoxin *h* was purified as previously described (Johnson *et al.*, 1987).

#### Germination of Wheat Seeds

15 Wheat seeds were sterilized by steeping in 50% (v/v) of Generic Bleach for 1 h at room temperature, followed by a thorough wash with distilled water. The sterilized seeds were placed in a plastic Petri dish on two layers of Whatman filter paper moistened with distilled water containing 100 µg/ml of chloramphenicol. Germination was continued at room temperature in a dark chamber for up to 5 days.

#### Preparation of Wheat Proteases

20 The endosperm (10-15 g fresh weight) isolated from 5-day germinated wheat seeds by excising the roots and shoots was extracted for 30 minutes at 4°C with 5 volumes of 200 mM sodium acetate, pH 4.6, containing 10 mM  $\beta$ -mercaptoethanol. The homogenate was centrifuged for 20 minutes at 48,000 g, 4°C. The pellet was discarded and the 25 supernatant fluid was fractionated with 30-70% ammonium sulfate. This fraction, which represented the protease preparation, was resuspended in a minimum volume of 20 mM sodium acetate, pH 4.6, containing 10 mM  $\beta$ -mercaptoethanol, and dialyzed against this buffer overnight at 4°C. When assayed with azocasein as substrate, the protease preparation had an optimal pH of about 4.6 and was stable for at least one week at 4°C.

#### Reduction and Proteolytic Susceptibility of Trypsin Inhibitors

30 Unless indicated, the reduction of the trypsin inhibitors (0.4 mg/ml) was carried out in 0.1 ml of 20 mM sodium phosphate buffer, pH 7.9 containing 10 mM EDTA at 30°C for 2 hours. The concentrations of thioredoxin, NTR, and NADPH were 0.024 mg/ml, 0.02 mg/ml, and 0.25 mM, respectively. With DTT as reductant, EDTA and components of the 35 NADP/thioredoxin system were omitted. Following reduction, aliquots of the inhibitor mixture were withdrawn either for determination of trypsin inhibitory activity or proteolytic susceptibility. In the subtilisin tests, the inhibitor mixture (50 µl) was directly mixed with subtilisin and incubated at room temperature for 1 hour. With the wheat protease preparation, the pH of the inhibitor mixture (50 µl) was first adjusted to 4.7 by mixing with 35 µl of 200 mM sodium acetate, pH 4.6; 10 µl of the wheat protease preparation was then added and incubation was continued for 2 hours at 37°C. To stop 40 digestion with subtilisin, 2 µl of 100 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µl of 10% SDS were added to the digestion mixture. With the plant protease preparation, digestion was stopped by adding an equal volume of SDS sample buffer [0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v)  $\beta$ -mercaptoethanol, and 0.02% (w/v) bromophenol blue]. Proteolytic products were analyzed by electrophoresis with 12% or 16% SDS polyacrylamide slab 45 gels (Laemmli, 1970). The dried slab gels were scanned with a laser densitometer (Pharmacia-LKB UltraScan XL) and the peak area of the KTI or BBTI protein band was obtained by integration with a Pharmacia GelScan XL software program.

#### Assays

50 Thioredoxin and NTR were assayed as previously described by Florencio *et al.* (1988). Trypsin activity was measured in 50 mM Tris-HCl, pH 7.9, by following the increase in absorbance at 253 nm with N-benzoyl-L-arginine ethyl ester as substrate (Mundy *et al.*, 1984) or by the release of azo dye into the trichloroacetic acid (TCA)-soluble fraction from azocasein substrate (see below). For trypsin inhibition assays, trypsin (5 to 10 µg) was preincubated with appropriate amounts of KTI or BBTI for 5 minutes at room temperature in 50 mM Tris-HCl, pH 7.9 and proteolytic activity was then 55 determined. While the two substrates yielded similar data, results are presented with only one substrate.

Wheat protease activity was measured by following the release of azo dye into TCA solution from azocasein substrate at pH 4.7. Fifty µl of wheat protease in a solution of 20 mM sodium acetate, pH 4.6, and 10 mM  $\beta$ -mercaptoethanol were added to 50 µl of 200 mM sodium acetate, pH 4.6, and 100 µl of 2% azocasein (in 20 mM sodium phosphate,

pH 7.0). Following 1-hour incubation at 37°C, 1 ml of 10% TCA was added and the mixture was allowed to stand for 10 minutes at room temperature. After centrifugation for 5 minutes in a microfuge (8000 g), 1 ml of the supernatant solution was withdrawn and mixed with 1 ml of 1 N NaOH. The absorbance was read at 440 nm. Protein concentration was determined with Bio-Rad reagent using bovine serum albumin as a standard (Bradford, 1976).

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## RESULTS

### Trypsin Inhibitory Activity

10 The 20 kDa Kunitz and 8 kDa Bowman-Birk trypsin inhibitors of soybean contain 2 and 7 disulfide groups, respectively (Birk, 1976; Wilson, 1988). Although their physiological functions have not been established, the two types of inhibitors have been extensively investigated owing to their wide distribution in legume seeds and their potential to cause nutritional disorders, e.g., hypertrophy and associated malfunctions of the pancreas. As shown in Tables I and II and described in previous Examples, KTI and BBTI are reduced specifically by the NADP/thioredoxin system from 15 either *E. coli* or plants. The reduced forms of glutathione and glutaredoxin (a thiol protein capable of replacing thioredoxin in certain animal and bacterial systems, but not known to occur in plants (Holmgren, 1985)) were without effect.

20 To determine the consequence of reduction by thioredoxin, the trypsin inhibitory activity of the oxidized and reduced forms of KTI and BBTI was compared. As shown in Table XI, preincubation with the NADP/thioredoxin system (NTS) for 2 hours at 30°C resulted in a substantial loss of trypsin inhibitory activity (i.e., there was an increase in trypsin 25 activity relative to the uninhibited control). More specifically, the NADP/thioredoxin system effected a 3- and 6-fold increase in trypsin activity for KTI and BBTI, respectively. Similar results were obtained with DTT, a nonphysiological substitute for thioredoxin, and with thioredoxin reduced by lipoic acid, a naturally occurring dithiol. Extended incubation with DTT alone (overnight at room temperature) led to complete or almost complete inactivation of both inhibitors (data not shown). Unlike DTT, lipoic acid did not reduce (inactivate) KTI and BBTI significantly in the absence of thioredoxin.

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TABLE XI

Changes in the Ability of Soybean Trypsin Inhibitors to Inhibit Trypsin Following Reduction by the NADP/Thioredoxin System, DTT or Reduced Lipoic Acid		
Treatment	Relative Trypsin Activity*	
	KTI	BBTI
No inhibitor	100	100
Inhibitor		
Oxidized	17.0	11.5
Reduced by NTS <sup>1</sup>	55.6	70.6
Reduced by DTT <sup>2</sup>	68.6	88.9
Reduced by LA/Trx h <sup>3</sup>	40.5	87.8

30 \*The specific activity of the uninhibited control trypsin was 0.018  $\Delta A_{253\text{nm}}/\mu\text{g}/\text{min}$  using N-benzoyl-L-arginine ethyl ester as substrate.

35 <sup>1</sup>Reduction by *E. coli* NTS (NADP/thioredoxin system) was conducted at 30°C for 2 hours.

40 <sup>2</sup>Reduction by DTT (1 mM) was conducted at 30°C for 1 hour.

45 <sup>3</sup>Reduction by lipoic acid (LA, 0.4 mM) and wheat thioredoxin h (Trx h) was conducted at 30°C for 1 hour. In the presence of lipoic acid alone (0.4 mM), trypsin activity was 20.0% for KTI and 12.5% for BBTI.

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55 Friedman and colleagues observed that heating soybean flour in the presence of sulfur reductants (sodium sulfite, N-acetyl-L-cysteine, reduced glutathione, or L-cysteine) inactivated trypsin inhibitors, presumably as a result of the reduction or interchange of disulfide groups with other proteins in soy flour (Friedman and Gumbmann, 1986; Friedman et al., 1982, 1984). Inactivation of the trypsin inhibitors by these reductants improved the digestibility and nutritive value of flours in tested rats (Friedman and Gumbman, 1986). Taken together with these earlier observations, the present findings demonstrate that disulfide bonds of both KTI and BBTI targeted by thioredoxin are important to maintenance

of trypsin inhibitory activity.

#### Heat Stability

5 Protease inhibitor proteins are typically stable to inactivation treatments such as heat. This stability is attributed, at least in part, to the cross-linking of disulfide bonds (Birk, 1976; Ryan, 1981). It is known that breaking the disulfide bonds by reduction decreases heat stability (Friedman *et al.*, 1982). The question arises as to whether reduction by thioredoxin yields similar results.

10 The results as shown in TABLE XII provide a positive answer to this question. When heated at 80°C for 15 minutes, about half of the original activity (Table XII). Oxidized BBTI was even more stable, retaining the bulk of its trypsin inhibitory activity after heating at 100°C for 25 minutes. Nonetheless, as with KTI, the reduced form of BBTI was fully inactivated by heat (Table XII). These results are consistent with prior observations (i) that KTI and BBTI show increased sensitivity to heat on reduction; and (ii) that pure BBTI in solution is more heat-stable than pure KTI in solution. The 15 reverse is true for flour (*i.e.*, KTI is more heat-stable than BBTI (Friedman *et al.*, 1982 and 1991; and DiPietro and Liener, 1989)).

TABLE XII

Heat Stability of the Kunitz and Bowman-Birk Trypsin Inhibitors: Oxidized and Following Reduction by the <i>E. coli</i> NADP/thioredoxin System		
Treatment	Relative Trypsin Activity*	
	KTI	BBTI
No inhibitor	100	100
Inhibitor, unheated		
Oxidized	26.6	9.4
Reduced	76.4	82.4
Inhibitor, heated 15 min at 80°C		
Oxidized	52.3	nd <sup>1</sup>
Reduced	98.7	nd
Inhibitor, heated 25 min at 100°C		
Oxidized	nd	17.2
Reduced	nd	98.4

\*The specific activity of trypsin was 0.319  $\Delta A_{440nm}/mg/min$  using azocasein as substrate. The temperatures used for inactivation were determined in initial experiments designed to show the heat stability of the trypsin inhibitors under our conditions.

<sup>1</sup>nd: not determined.

#### Protease Susceptibility

50 To test whether the reduced forms of KTI and BBTI show decreased stability to proteases other than trypsin, both the reduced and oxidized forms of KTI and BBTI were incubated with a wheat protease preparation or with subtilisin and the proteolytic products were analyzed by SDS-PAGE. The extent of proteolysis was determined by measuring the abundance of intact protein on SDS gels by laser densitometer. When tested with a protease preparation from 5-day germinated wheat seeds, the oxidized form of the Kunitz inhibitor was almost completely resistant to digestion whereas the thioredoxin-reduced form was susceptible to protease. As shown in Table XIII, about 80% of KTI was degraded in a reaction that depended on all components of the NADP/thioredoxin system (NTS). BBTI showed the same pattern except that the oxidized protein showed greater proteolytic susceptibility relative to KTI. Similar effects were observed

with both inhibitors when the plant protease preparation was replaced by subtilisin (data not shown). The nature of the proteolytic reaction was not investigated, but it is noted that peptide products were not detected on SDS gels.

TABLE XIII

Effect of Thioredoxin-linked Reduction on the Susceptibility of Kunitz and Bowman-Birk Trypsin Inhibitors to Proteolysis by a Plant Protease Preparation <sup>1</sup>		
Treatment	Relative Abundance <sup>2</sup>	
	KTI	BBTI
No protease	100	100
Protease		
No reduction system	97.9	67.2
<i>E. coli</i> NTS <sup>3</sup>	22.1	16.0
NTS minus thioredoxin	90.2	nd <sup>4</sup>
NTS minus NADPH	97.7	nd
NTS minus NTR	97.9	nd

<sup>1</sup>Following reduction by *E. coli* thioredoxin system at 30°C for 2 hours, pH was adjusted to 4.7 by addition of 200 mM sodium acetate, pH 4.6. Wheat protease preparation was then added and incubated at 37°C for 2 hours, followed by SDS-PAGE analyses.

<sup>2</sup>Determined by laser densitometer.

<sup>3</sup>NTS: NADP/thioredoxin system.

<sup>4</sup>nd: not determined.

This Example shows that reduction by thioredoxin, or dithiothreitol (DTT), leads to inactivation of both proteins and to an increase in their heat and protease susceptibility. The results indicate that thioredoxin-linked reduction of the inhibitor proteins is relevant both to their industrial processing and to seed germination.

These results confirm the conclusion that disulfide bonds are essential for the trypsin inhibitory activity of KTI and BBTI (Birk, 1985; Friedman and Gumbmann, 1986; Friedman *et al.*, 1982, 1984). These studies also show that reduction (inactivation) can take place under physiological conditions (*i.e.*, at low temperature with NADPH-reduced thioredoxin). The ability to inactivate the trypsin inhibitors at lower temperatures provides a potential method for full inactivation of both trypsin inhibitors, thereby improving the quality of soybean products and saving energy. The need for a method for the complete inactivation of KTI is significant since 20% of its activity is consistently retained in soy flour under conditions in which BBTI is fully inactivated (Friedman *et al.*, 1991).

The present results also add new information on the protease susceptibility of KTI and BBTI. Their increase in protease susceptibility following reduction suggests that, if exposed to the protease inhibitors during seed germination, the NADP/thioredoxin system could serve as a mechanism by which the inhibitor proteins are modified (inactivated) and eventually degraded (Baumgartner and Chrispeels, 1976; Chrispeels and Baumgartner, 1978; Orf *et al.*, 1977; Wilson, 1988; Yoshikawa *et al.*, 1979). As stated previously, there is evidence that the NADP-thioredoxin system plays a similar role in mobilizing proteins during the germination of wheat seeds.

#### EXAMPLE 20

##### Reduction of Castor Seed 2S Albumin Protein by Thioredoxin

The results of the following study of sulphhydryl agents to reduce the 2S protein from castor seed (Sharief and Li, 1982; Youle and Huang, 1978) shows that thioredoxin actively reduces intramolecular disulfides of the 2S large subunit but not the intermolecular disulfides joining the two subunits.

Materials and MethodsMaterials

5 Seeds of castor (*Ricinus communis* L. var Hale) were obtained from Bothwell Enterprises, Plainview, TX). Bio-chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). *E. coli* thioredoxin and NTR were isolated from cells transformed to overexpress each protein. The thioredoxin strain containing the recombinant plasmid pFPI, was kindly provided by Dr. J.-P. Jacquot (de La Mott-Guery *et al.* 1991). The strain containing the recombinant plasmid, pPMR21, was kindly provided by Drs. Marjorie Russel and Peter Model (Russel and Model, 1988). Thioredoxin and NTR were purified by the respective procedures of de La Mott-Guery *et al.* (1991) and Florencio *et al.* (1988). Reagents for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). Monobromobimane (mBBr) or Thiolite was obtained from Calbiochem (San Diego, CA). Other chemicals were obtained from commercial sources and were of the highest quality available. NADP-malate dehydrogenase and fructose-1,6-bisphosphatase were purified from leaves of corn (Jacquot *et al.* 1981) and spinach (Nishizawa *et al.* 1982), respectively. Thioredoxin h was isolated from wheat seeds by following the procedure devised for the spinach protein (Florencio *et al.* 1988). Glutathione reductase was prepared from spinach leaves (Florencio *et al.* 1988).

Isolation of Protein Bodies

20 Protein bodies were isolated by a nonaqueous method (Yatsu and Jacks, 1968). Shelled dry castor seeds, 15 g, were blended with 40 ml of glycerol for 30 sec in a Waring blender. The mixture was filtered through four layers of nylon cloth. The crude extract was centrifuged at 272 x g for 5 min in a Beckman J2-21M centrifuge using a JS-20 rotor. After centrifugation, the supernatant fraction was collected and centrifuged 20 min at 41,400 x g. The pellet, containing the protein bodies, was resuspended in 10 ml glycerol and centrifuged as before (41,400 x g for 20 min) collecting the pellet. This washing step was repeated twice. The soluble ("matrix") fraction was obtained by extracting the pellet with 3 ml of 100 mM Tris-HCl buffer (pH 8.5). The remaining insoluble ("crystalloid") fraction, collected by centrifugation as before, was extracted with 3 ml of 6M urea in 100 mM Tris-HCl buffer (pH 8.5).

25 Protein Purification Procedure

30 The 2S protein was prepared by a modification of the method of Tully and Beevers (1976). The matrix protein fraction was applied to a DEAE-cellulose (DE-52) column equilibrated with 5 mM Tris-HCl buffer, pH 8.5 (Buffer A) and eluted with a 0 to 300 mM NaCl gradient in buffer A. Fractions containing the 2S protein were pooled and concentrated by freeze drying. The concentrated fraction was applied to a Pharmacia FPLC Superose-12 (HR 10/30) column equilibrated with buffer A containing 150 mM NaCl. The fraction containing 2S protein from the Superose-12 column was applied to an FPLC Mono Q HR 5/5 column equilibrated with buffer A. The column was eluted sequentially with 3 ml of buffer A, 20 ml of a linear gradient of 0 to 300 mM NaCl in buffer A and finally with buffer A containing 1 M NaCl. The 2S protein purified by this method was free of contaminants in SDS polyacrylamide gels stained with Coomassie blue (Kobrehel *et al.*, 1991).

Analytical Methods

40 Reduction of proteins was monitored by the monobromobimane (mBBr)/SDS polyacrylamide gel electrophoresis procedure of Crawford *et al.* (1989). Labeled proteins were quantified as described previously in the "Reduction of Cereal Proteins, Materials and Methods" section. Protein was determined by the method of Bradford (1976).

Enzyme Assays/Reduction Experiments

45 The Wada *et al.*, 1981 protocol was used for assaying NADP-malate dehydrogenase and fructose 1,6 bisphosphatase in the presence of thioredoxin and 2S protein. Assays were conducted under conditions in which the amount of added thioredoxin was sufficient to reduce the castor 2S protein but insufficient to activate the target enzyme appreciably. All assays were at 25°C. Unless otherwise indicated, the thioredoxin and NTR used were from *E. coli*. The 2S protein was monitored during purification by mBBr/SDS-polyacrylamide gel electrophoresis following its reduction by dithiothreitol and *E. coli* thioredoxin (Crawford *et al.*, 1989; Kobrehel *et al.*, 1991).

50 Fig. 39 represents the reduction of the matrix and crystalloid proteins from castor seed as determined by mBBr/SDS-polyacrylamide gel electrophoresis procedure. 1 and 7, Control: no addition; 2 and 8, GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach leaves) and NADPH; 3 and 9, NGS: NADPH, reduced glutathione, glutathione reductase (from spinach leaves) and glutaredoxin from *E. coli*; 4 and 10, NTS: NADPH, NTR, and

thioredoxin (both proteins from *E. coli*); 5 and 11, NADPH; 6 and 12, NADPH and *E. coli* NTR. Reactions were carried out in 100 mM Tris-HCl buffer, pH 7.8. As indicated, 0.7 µg NTR and 1 µg thioredoxin were added to 70 µl of this buffer containing 1 mM NADPH and target protein: 8 µg matrix protein for treatments 1-6 and 10 µg crystalloid protein for treatments 7-12. Assays with glutathione were performed similarly, but at a final concentration of 2 mM, 1.4 µg glutathione reductase, 1 µg glutaredoxin, and 1.5 mM NADPH. Reaction time was 20 min.

Fig. 40 represents the specificity of thioredoxin for reducing the disulfide bonds of castor seed 2S protein. (1) Control (no addition); (2) Control + NTS (same conditions as in Fig. 39); (3) Control (heated 3 min at 100°C); (4) Control + 2 mM DTT (heated 3 min at 100°C). The samples containing 5 µg 2S protein and the indicated additions were incubated for 20 min in 30 mM Tris-HCl (pH 7.8). mBBr, 80 nmol, was then added and the reaction continued for another 15 min prior to analysis by the mBBr/SDS polyacrylamide gel electrophoresis procedure.

### Results

The castor storage proteins, which are retained within a protein body during seed maturation, can be separated into two fractions on the basis of their solubility. The more soluble proteins are housed in the protein body outer section ("matrix") and the less soluble in the inner ("crystalloid"). In the current study, the matrix and crystalloid components were isolated to determine their ability to undergo reduction by cellular thiols, viz., glutathione, glutaredoxin and thioredoxin. Glutaredoxin, a 12 kDa protein with a catalytically active thiol group, can replace thioredoxin in certain enzymic reactions of bacteria and animals (Holmgren *et al.* 1985) but is not known to occur in plants.

Fig. 39 shows that, while a number of storage proteins of castor seed were reduced by the thiols tested, only a low molecular weight protein, corresponding to the large subunit of the 2S protein of the matrix, showed strict specificity for thioredoxin. Certain higher molecular weight proteins of the crystalloid fraction underwent reduction, but in those cases there was little difference between glutaredoxin and thioredoxin (Fig. 39). The castor seed 2S large subunit thus appeared to resemble cystine-containing proteins previously discussed in undergoing thioredoxin-specific reduction. These experiments were designed to confirm this specificity and to elucidate certain properties of the reduced protein. As expected, owing to lack of disulfide groups, the 2S small subunit showed essentially no reaction with mBBr with any of the reductants tested.

When its fluorescent band was monitored by laser densitometry, the reduction of the castor seed 2S large subunit was found to depend on all components of the NADP/thioredoxin system (NADPH, NTR and thioredoxin) (Table XIV). As for other thioredoxin-linked proteins (including chloroplast enzymes), the thioredoxin active in reduction of the 2S large subunit could be reduced either chemically with dithiothreitol (DTT) or enzymatically with NADPH and NTR. The extent of reduction by the NADP thioredoxin system, DTT alone, and DTT + thioredoxin was 84%, 67% and 90%, respectively, after 20 min at 25°C. Similar, though generally extensive reduction was observed with the disulfide proteins discussed above (Johnson *et al.* 1987). As with the other seed proteins, native wheat thioredoxin *h* and *E. coli* thioredoxins could be used interchangeably in the reduction of the 2S protein by DTT (data not shown).

TABLE XIV

Extent of reduction of the castor castor seed 2S protein by different sulphydryl reductants. Reaction conditions as in Fig. 39. A reduction of 100% corresponds to that obtained when the 2S protein was heated for 3 min in the presence of 2% SDS and 2.5% β-mercaptoethanol. NTS: NADPH, NTR, and thioredoxin (both proteins from <i>E. coli</i> ); GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach leaves) and NADPH; NGS: NADPH, reduced glutathione, glutathione reductase (from spinach leaves) and glutaredoxin (from <i>E. coli</i> ).	
Treatment	Relative Reduction, %
Control	0
NADP/thioredoxin system, complete	84
" minus thioredoxin	0
" minus NADPH	0
" minus NTR	0
Reduced glutathione	0
NADP/glutaredoxin system, complete	0
DTT	67
DTT + Thioredoxin	90

The capability of thioredoxin to reduce the castor seed 2S protein was also evident in enzyme activation assays. Here, the protein targeted by thioredoxin (in this case 2S) is used to activate a thioredoxin-linked enzyme of chloroplasts, NADP-malate dehydrogenase or fructose 1,6-bisphosphatase. As with most of the proteins examined so far, the 2S protein more effectively activated NADP-malate dehydrogenase and showed little activity with the fructose bisphosphatase (2.6 vs. 0.0 nmoles/min/mg protein).

The castor seed 2S protein contains inter- as well as intramolecular disulfides. The 2S protein thus provides an opportunity to determine the specificity of thioredoxin for these two types of bonds. To this end, the castor seed 2S protein was reduced (i) enzymically with the NADP/thioredoxin system at room temperature, and (ii) chemically with DTT at 100°C. Following reaction with mBBR the reduced proteins were analyzed by SDS-polyacrylamide gel electrophoresis carried out without additional sulfhydryl agent. The results (Fig. 40) indicate that while thioredoxin actively reduced intramolecular disulfides, it was much less effective with intermolecular disulfides.

The present results extend the role of thioredoxin to the reduction of the 2S protein of castor seed, an oil producing plant. Thioredoxin specifically reduced the intramolecular disulfides of the large subunit of the 2S protein and showed little activity for the intermolecular disulfides joining the large and small subunits. Based on the results with the trypsin inhibitors of soybean, it is clear that reduction of intramolecular disulfides by thioredoxin markedly increases the susceptibility of disulfide proteins to proteolysis (Jiao *et al.* 1992a). It, however, remains to be seen whether reduction of the 2S protein takes place prior to its proteolytic degradation (Youle and Huang, 1978) as appears to be the case for the major storage proteins of wheat. A related question raised by this work is whether the 2S protein of castor, as well as other oil producing plants such as brazil nut (Altenbach *et al.*, 1987; Ampe *et al.*, 1986), has a function in addition to that of a storage protein.

#### EXAMPLE 21

##### Thioredoxin-Dependent Deinhibition of Pullulanase of Cereals by Inactivation of a Specific Inhibitor Protein

##### Assay of Pullulanase

###### 1. Standard curve of maltotriose:

A series of concentrations of maltotriose (0 to 2 mg) in 0.1 to 0.2 ml water or buffer were made in microfuge tubes. To this was added 0.2 ml of dinitrosalicylic acid (DA) reagent (mix 1 g of DA, 30 g of sodium potassium tartrate, and 20 ml of 2N NaOH with water to final volume of 100 ml). The reagents were dissolved in a warm water bath. The mixture was heated at 100°C for 5 min and cooled down in a water bath (room temperature). Each sample was transferred to a glass tube that contained 2 ml of water. Read  $A_{493}$  vs water.  $\Delta A_{493}[A_{493}$  of sample containing maltotriose was subtracted from  $A_{493}$  of the blank (no maltotriose)] was plotted against maltotriose concentrations.

###### 2. Pullulanase activity assay

Pullulanase activity is measured as the release of reducing sugar from the substrate pullulan. Typically 10-100  $\mu$ l of pullulanase sample (in 20 mM Tris-HCl, pH 7.5, or in 5-20 acetate-NA, pH 4.6) was mixed with 25-100  $\mu$ l of 200 mM Acetate-NA, pH 5.5 (this buffer serves to bring final pH of the assay to 5.5) and 10-20  $\mu$ l of 2% (w/v) pullulan. The mixture was incubated at 37°C for 30 to 120 min, depending on the activity of pullulanase. The reaction was stopped by adding 200  $\mu$ l of DA reagent. Reducing sugar was then determined as above.

###### 45 Note:

- When a crude extract of pullulanase obtained by the dialysis of crude extracts or pullulanase obtained from a dialyzed 30-60% ammonium sulfate fraction is used as a pullulanase source, it must be thoroughly dialysed before assay because there are reduced sugars in the crude extract. In other words the background of crude pullulanase samples from dialysed crude extracts or a dialysed 30-60% ammonium sulfate fraction is very high. In this case, the blank is made as follows: 200  $\mu$ l of DA reagent are added first, followed by the addition of enzyme sample, pullulan and buffer.
- When final concentrations of DTT (or  $\beta$ -mercaptoethanol (MET) or GSH) are higher than 2 mM in the assay mixture, the  $OD_{493}$  values will be greater than those of the minus-DTT (MET, GSH) samples. DTT (MET, GSH) should be added to the blank, samples without DTT during assay at the end of the reaction. Care should be taken to make sure the final concentration of DTT in the assay mixture is below 2 mM.

Purification of Pullulanase Inhibitor Extraction and Ammonium Sulfate Fractionation

200 g of barley malt was ground to fine powder with an electric coffee grinder and extracted with 600 ml of 5% (w/v) NaCl for 3 h at 30°C. Following centrifugation at 30,000 g and at 4°C for 25 min, the supernatant was fractionated by 5 precipitation with solid ammonium sulfate. Proteins precipitated between 30% and 60% saturated ammonium sulfate were dissolved in a minimum volume of 20 mM Tris HCl, pH 7.5, and dialyzed against this buffer at 4°C overnight.

DE52 Chromatography

10 The dialysed sample was centrifuged to remove insoluble materials and the supernatant adjusted to pH 4.6 with 2N formic acid. After pelleting the acid-denatured protein, the supernatant was readjusted to pH 7.5 with NH<sub>4</sub>OH and loaded onto a DE52 column (2.5 x 26 cm) equilibrated with 20 mM Tris-HCl, pH 7.5. Following wash with 80 ml of the above buffer, the column was eluted with a linear 0-500 mM Tris-HCl, pH 7.5. Fractions of 6.7 ml were collected. Pullulanase was eluted at about 325 mM NaCl and its inhibitor came off at about 100 mM NaCl. Pullulanase was further purified through CM32 (20 mM sodium acetate, pH 4.6) and Sephadryl-200 HR (30 mM Tris-HCl, pH 7.5, containing 200 15 mM NaCl and 1 mM EDTA) chromatography. Pullulanase inhibitor protein was purified as described below.

CM32 Chromatography

20 The pullulanase inhibitor sample (about 90 ml) from the DE52 step was placed in a 150-ml flask and incubated at 70°C water-bath for 20 min. Following centrifugation, the clarified sample was then adjusted to pH 4.6 with 2N formic acid and dialyzed against 20 mM sodium acetate, pH 4.6. The precipitate formed during dialysis was removed by centrifugation and the supernatant was chromatographed on a CM32 column (2.5 X 6 cm) equilibrated with 20 mM sodium acetate, pH 4.6. Proteins were eluted with a linear 0-0.4 M NaCl in 200 ml of 20 mM sodium acetate, pH 4.6. Fractions 25 (5.0 ml/fraction) containing pullulanase inhibitory activity were pooled, dialyzed, and rechromatographed on a CM32 column (2.5 x 6 cm) with a linear 0.2-1 M NaCl gradient in 200 ml of 20 mM sodium acetate, pH 4.0.

Sephadex G-75 Filtration

30 Pullulanase inhibitor fractions from the second CM32 chromatography step were concentrated in a dialysis bag against solid sucrose and then separated by a Sephadex G-75 column (2.5 x 85 cm) equilibrated with 30 mM Tris-HCl, pH 7.5, containing 200 mM Na Cl and 1 mM EDTA. Fractions (3.6 ml/fraction) showing pullulanase inhibitory activity were pooled, concentrated by dialysis against solid sucrose, and then dialysed against 10 mM Tris-HCl, pH 7.5.

Identification and Purification of Pullulanase Inhibitor

35 During germination, starch is converted to glucose by  $\alpha$ -,  $\beta$ -amylases, and pullulanase (also called debranching enzyme, R-enzyme). While extensive studies have been conducted for the regulation of amylases, little is known about the regulation of pullulanase in seeds. Yamada (Yamada, J. (1981) Carbohydrate Research 90:153-157) reported that 40 incubation of cereal flours with reductants (e.g., DTT) or proteases (e.g., trypsin) led to an activation of pullulanase activity, suggesting that reduction or proteolysis might be a mechanism by which pullulanase is activated during germination. Like in rice flour, pullulanase extracts from germinated wheat seeds or from barley malt showed lower activity, and were activated 3 to 5-fold by preincubation with DTT for 20 to 30 min. However, following purification of the crude 45 extract (a dialysate of 30-60% ammonium sulfate fraction) by anion or cation exchange chromatography, the total pullulanase activity increased 2 to 3-fold over that of the sample applied to the column when assayed without preincubation with DTT, and DTT has no or little effect on pullulanase. One possibility was that pullulanase might be activated by proteolysis during the process of purification, since germinated wheat seeds or barley malt show high protease activity. If this was the case, addition of protease inhibitor cocktail would prevent pullulanase activation during purification. In contrast to this point, many experiments with protease inhibitors failed to prove this. Another possibility was that there is an 50 inhibitor that is precipitated by ammonium sulfate and inhibits pullulanase. The role of DTT is to reduce and thus inactivate this protein inhibitor, leading to activation of pullulanase. Along this line, the 30-60% ammonium sulfate fraction from barley malt was applied to a DE52 column (2.5 x 26 cm) equilibrated with 20 mM Tris-CHI, pH 7.5 (Fig. 41). Following elution with a linear salt gradient, "deinhibited" ("activated") pullulanase was identified as a protein peak coming off at about 325 mM NaCl (from fraction numbers 44 to 60). Assay of pullulanase activity in the preincubation mixture 55 consisting of 50  $\mu$ l of the peak pullulanase activity fraction (fraction number 45) with 50  $\mu$ l of other protein fractions indicated that a protein peak that showed pullulanase inhibitory activity was eluted from the DE52 column by about 100 mM NaCl between fraction numbers 8 to 25 (Fig. 41).

The pullulanase inhibitor sample was further purified by two consecutive cation exchange chromatography steps

with CM32 at pH 4.6 (Fig. 42) and 4.0 (Fig. 43) and filtration with Sephadex G-75 (Fig. 44).

Properties of Pullulanase Inhibitor

5 Preliminary experiments showed that pullulanase inhibitor protein is resistant to treatment of 70°C for 10 min and pH 4.0. Based on the profile of Sephadex G-75 gel filtration and SDS-PAGE, pullulanase inhibitor has a molecular weight between 8 to 15 kDa  $\pm$  2 kDa. The study further showed that the protein contains thioredoxin-reducible (S-S) bonds.

10 These studies, as shown in Table XV, found that the ubiquitous dithiol protein, thioredoxin, serves as a specific reductant for a previously unknown disulfide-containing protein that inhibits pullulanase of barley and wheat endosperm.

TABLE XV

Activity Change in Pullulanase Inhibitor Protein Following Reduction by NADP/Thioredoxin System	
Treatment	Relative Pullulanase Activity
No inhibitor	100
Inhibitor	
Oxidized	30.1
Reduced by DTT	46.1
Reduced by <i>E. coli</i> Trx/DTT	95.1
Reduced by <i>E. coli</i> NTS	40.4
Reduced by GSH/NADPH/GR	33.6

30

Reduction of the inhibitor protein eliminated its ability to inhibit pullulanase, thereby rendering the pullulanase enzyme active. These studies as shown in Table XV illustrate that it is possible to render the pullulanase enzyme active with a physiological system consisting of NADPH, NADP-thioredoxin reductase (NTR) and thioredoxin (the NADP/thioredoxin system) as well as with thioredoxin (Trx) and dithiothreitol. These findings also elucidate how reductive activation of pullulanase takes place (i.e., that a specific (previously unknown) inhibitor is reduced and thereby inactivated, so that the enzyme becomes active). The thioredoxin active in this reaction can be obtained from several sources such as *E. coli* or seed endosperm (thioredoxin h). The role of thioredoxin in reductively inactivating the inhibitor protein (I) and deinhibiting the pullulanase enzyme (E) is given in Equations 1 and 2.



45 In summary, the crude endosperm extracts were fractionated by column chromatography procedures. These steps served to separate the protein inhibitor from the pululanase enzyme. The inhibitor protein was then highly purified by several steps. By use of the mBBr/SDS-PAGE procedure, it was determined that disulfide group(s) of the new protein are specifically reduced by thioredoxin and that the reduced protein loses its ability to inhibit pullulanase. Like certain other disulfide proteins of seeds (e.g., the Kunitz and Bowman-Birk trypsin inhibitors of soybean), the pullulanase inhibitor protein showed the capability to activate chloroplast NADP-malate dehydrogenase. In these experiments, dithiothreitol was used to reduce thioredoxin, which in turn reduced inhibitor and thereby activated the dehydrogenase enzyme.

EXAMPLE 22

55 Engineering of Yeast Cells to Overexpress Thioredoxin and NADP-Thioredoxin Reductase

The two *Saccharomyces cerevisiae* thioredoxin genes (Muller, E.G.D. (1991), *J. Biol. Chem.* **266**:9194-9202), TRX1 and TRX2, are cloned in high copy number episomal vectors, an example of which is YEp24, under the control

of strong constitutive promoter elements, examples of which are the glycolytic promoters for the glyceraldehyde-3-P dehydrogenase, enolase, or phosphoglycerate kinase genes. Recombinant constructs are assessed for the overexpression of thioredoxin by quantitative Western blotting methods using an antithioredoxin rabbit antiserum (Muller, E.G.D., et al. (1989), *J. Biol. Chem.* 264:4008-4014), to select the optimal combination of thioredoxin genes and promoter elements. The cells with the optimal thioredoxin overexpression system are used as a source of thioredoxin for dough improvement.

The NADP-thioredoxin reductase gene is cloned by preparing an oligonucleotide probe deduced from its amino terminal sequence. The enzyme is prepared from yeast cells by following a modification of the procedure devised for spinach leaves (Florencio, F.J., et al. (1988), *Arch. Biochem. Biophys.* 266:496-507). The amino terminus of the pure reductase enzyme is determined by microsequencing by automated Edman degradation with an Applied Biosystems gas-phase protein sequencer. On the basis of this sequence, and relying on codon usage in yeast, a 20-base 24-fold degenerate DNA probe is prepared. The probe is hybridized to isolated yeast DNA cleaved with *Eco*RI and *Pst*I by Southern blot analysis. The most actively region is extracted from the agarose gels and introduced into a pUC19 plasmid vector (Szekeres, M., et al. (1991), *J. Bacteriol.* 173:1821-1823). Following transformation, plasmid-containing *E. coli* colonies are screened by colony hybridization using the labeled oligonucleotide probe (Vogeli, G., et al. (1987), *Methods Enzymol.* 152:407-415). The clone is identified as carrying the gene for NADP-thioredoxin reductase by sequencing the DNA as given in Szekeres, et al. above. Once identified, the NADP-thioredoxin reductase gene is overexpressed in yeast as described above for the TRX1 and TRX2 yeast thioredoxin genes. The yeast cells which overexpress NADP-thioredoxin reductase are used as a source of reductase to improve dough quality.

#### EXAMPLE 23

##### Improvement in Dough Quality Using Genetically Engineered Yeast Cells

*Saccharomyces cerevisiae* cells engineered to overexpress the two yeast thioredoxins and the yeast NADP-thioredoxin reductase as set forth in Example 23 are lysed by an established procedure such as sonication and then freeze dried. The dried cells from the cultures overexpressing thioredoxin and NADP-thioredoxin reductase are combined and then used to supplement flour to improve its dough quality. Two-tenths gram of the combined lysed dried cells are added together with about 300 to about 500 nanomoles NADPH to 1 M Tris-HCl buffer, pH 7.9, to give 5.25 ml of 30 mM Tris-HCl. The reaction is carried out in a microfarinograph at 30°C as described in Example 14. An improvement in dough quality is observed which is similar to the improvement shown in Example 14.

#### EXAMPLE 24

##### Improvement of Gluten

The positive effects of the NADP/thioredoxin system on dough quality presents the option of applying this system to flour in the preparation of gluten. The purpose is to alter the yield and the properties of gluten, thereby enhancing its technological value: (1) by obtaining stronger gluts (increased elasticity, improved extensibility); (2) by increasing gluten yield by capturing soluble proteins, reduced by the NADP-thioredoxin system, in the protein network, thereby preventing them from being washed out during the production of gluten. In this procedure (using 10 g flour), 0.2 µg *E. coli* thioredoxin, 0.1 µg *E. coli* NADP-thioredoxin reductase and 300 to 500 nanomoles NADPH are added together with 1 M Tris-HCl, pH 7.9, buffer to give 5.25 ml of 30 mM Tris-HCl. The gluten is made at room temperature according to the common lixiviation method. The yield of the gluten is determined by weight and the strength of the gluten is determined by the classical manual stretch method. The gluten product which are obtained by this treatment with the NADP/thioredoxin system is used as an additive in flour or other grain.

#### EXAMPLE 25

##### Method of Producing Dough from a Non-wheat or Rye Flour

For this test (using 10 gm of milled flour from corn, rice or sorghum), 0.2 µg *E. coli* thioredoxin, 0.1 µg *E. coli* NADP-thioredoxin reductase and 500 nanomoles NADPH are added together with 1 M Tris-HCl, pH 7.9, buffer to give 5.25 ml of 30 mM Tris-HCl. The reaction is carried out by mixing the 10 gm of milled flour with the enzyme system in a microfarinograph at 30°C. The farinograph measurements show wheat-like dough characteristics by the added NADP-thioredoxin system. In the controls without the enzyme system, no microfarinograph reading is possible because the mixture fails to form a dough. The dough which is formed is persistent and its consistency is maintained throughout the run. The end product is similar to the network formed in dough derived from wheat.

REDUCTION OF ANIMAL TOXINS

The invention provides a method for chemically reducing toxicity causing proteins contained in bee, scorpion and snake venome and thereby altering the biological activity of the venoms well as reducing the toxicity of animal toxins specifically snake neurotoxins by means of thiol redox (SH) agents namely a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin or DTT. The reduction of the thioredoxin occurs preferably via the NADP-thioredoxin system (NTS). As stated previously, the NTS comprises NADPH, NADP-thioredoxin reductase (NTR) and a thioredoxin.

The term "thiol Redox agent" has been used sometimes in the literature to denote both an agent in the nonreduced state and also in the reduced or sulfhydryl (SH) state. As defined herein the term "thiol redox (SH) agent" means a reduced thiol redox protein or synthetically prepared agent such as DTT.

The reduction of the neurotoxin may take place in a medium that is liquid such as blood, lymph or a buffer, etc. or in a medium that is solid such as cells or other living tissue. As used herein the term "liquid" by itself does not refer to a biological fluid present in an individual.

Presumably the proficiency of the thiol redox (SH) agents to inactivate the venom *in vitro* and to detoxify the venom in individuals depends upon the ability of the agents of the invention to reduce the intramolecular disulfide bonds in these toxicity causing venom components.

All snake neurotoxins, both presynaptic and postsynaptic can be reduced and at least partially inactivated *in vitro* by the thiol redox (SH) agents of the invention. Snake neurotoxins inactivated *in vitro* according to the invention are useful as antigens in the preparation of antivenoms. The neurotoxins are inactivated preferably by incubation with a thiol redox (SH) agent in an appropriate buffer. The preferred buffer is Tris-HCl buffer but other buffers such as phosphate buffer may be used. The preferred thiol redox (SH) agent is a reduced thioredoxin.

Effective amounts for inactivating snake neurotoxins range from about 0.1  $\mu$ g to 5.0  $\mu$ g, preferably about 0.5  $\mu$ g to 1.0  $\mu$ g, of a reduced thioredoxin; from about 1 nanomole to 20 nanomoles, preferably from 5 nanomoles to 15 nanomoles, of reduced lipoic acid in the presence of about 1.0  $\mu$ g of a thioredoxin and from about 10 nanomoles to 200 nanomoles, preferably 50 nanomoles to 100 nanomoles, of reduced DTT (preferably in the presence of about 1.0  $\mu$ g of a thioredoxin) for every 10  $\mu$ g of snake neurotoxin in a volume of 100  $\mu$ l.

The effective amounts for inactivating a snake neurotoxin using the components in the NADP-thioredoxin system range from about 0.1  $\mu$ g to 5.0  $\mu$ g, preferably about 0.5  $\mu$ g to 1.0  $\mu$ g, of thioredoxin; from about 0.1  $\mu$ g to 2.0  $\mu$ g, preferably from 0.2  $\mu$ g to 1.0  $\mu$ g, of NTR and from about 0.05 micromoles to 0.5 micromoles, preferably about 0.1 micromoles to 0.25 micromoles, of NADPH for every 10  $\mu$ g of snake neurotoxin in a volume of 100  $\mu$ l.

Upon inactivation the buffer containing the inactivated neurotoxin and thiol redox (SH) agent, etc. may be injected into an animal such as a horse to produce an antivenom or prior to injection it may be further treated with heat or formaldehyde.

The thiol redox (SH) agents of the invention may also be used to treat individuals who are suffering the effects of neurotoxicity caused by a venomous snake bite. The preferred method of administering the reduced thiol redox (SH) agent to the individual is by multiple subcutaneous injections around the snake bite.

Of course the correct amount of a thiol redox (SH) agent used to detoxify a neurotoxin in an individual will depend upon the amount of toxin the individual actually received from the bite. However, effective amounts for detoxifying or reducing the toxicity of snake neurotoxins in mice usually range from about 0.01  $\mu$ g to 0.3  $\mu$ g, preferably about 0.02  $\mu$ g to 0.05  $\mu$ g, of a reduced thioredoxin; from about 0.1 nanomole to 3.0 nanomoles, preferably from 0.2 nanomole to 1.0 nanomoles, preferably from 2.0 nanomoles to 5.0 nanomoles, of DTT, preferably in the presence of 0.05  $\mu$ g of a thioredoxin, for every gm of mouse body weight.

The effective amounts for detoxifying a snake neurotoxin in a mouse using the components of the NADP-thioredoxin system range from about 0.01  $\mu$ g to 0.3  $\mu$ g, preferably about 0.02  $\mu$ g to 0.05  $\mu$ g of a thioredoxin; from about 0.005  $\mu$ g to 0.12  $\mu$ g, preferably from 0.01  $\mu$ g to 0.025  $\mu$ g, of NTR and from about 5 nanomoles to 30 nanomoles, preferably 10 nanomoles to 15 nanomoles, NADPH for every gm of mouse body weight.

The preferred method of administering the NTS to an individual is also by multiple subcutaneous injections. The preferred thiol redox agent for human use is human thioredoxin administered via the NADP-thioredoxin system or with lipoic acid or DTT.

A partial list of the venomous snakes which produce the neurotoxins which can be inactivated or detoxified by the methods of this invention appears on pages 529-555 of Chippaur, J.-P., et al. (1991) Reptile Venoms and Toxins, A.T. Tu, ed., Marcel Dekker, Inc., which is herein incorporated by reference.

Other features and advantages of the invention with respect to inactivating and detoxifying venome can be ascertained from the following examples.

EXAMPLE 26Reduction Studies of Bee, Scorpion and Snake Venoms and Labeling with mBBr

5 Reactions were carried out with 50 µg venom (final volume of 100 µl) in 30 mM Tris-CHI buffer pH 7.9 containing the following protease inhibitors: phenylmethylsulfonyl fluoride (PMSF), leupeptin and pepstatin (final concentrations used in the assay respectively: 100 µM, 1 µM and 1 µM). With NADPH as a reductant, the mixture also contained 4 µg thioredoxin, 3.5 µg NTR (both from *E. coli*) and 12.5 mM NADPH. When thioredoxin (4 µg, *E. coli* or human) was reduced by DTT, NADPH and NTR were omitted and DTT was added to 0.5 mM. Assays with GSH were performed similarly but at a final concentration of 5 mM and in the presence of 1.5 µg glutathione reductase and 12.5 mM NADPH. The mixture was incubated for 20 min at room temperature, mBBr was then added to 1.5 mM and the reaction was continued for 15 min at room temperature. The reaction was stopped and excess mBBr derivitized by adding 10 µl of 100 mM β-mercaptoethanol, 5 µl of 20% SDS and 10 µl of 50% glycerol. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis as previously described.

10 15 The same experiment with the NADP-thioredoxin system was performed without adding protease inhibitors.

10 The extent of the reduction of the bee, scorpion and snake venoms by different reductants described above is shown in Figs. 45, 46 and 47, respectively. Figs. 45, 46 and 47 represent the results of the reduction studies of different venoms (SDS-Polyacrylamide gel/mBBr procedure). After 20 min incubation at room temperature with different reductants and in the presence of protease inhibitors, the samples were derivatized with mBBr and separated by electrophoresis and fluorescence was determined. (Fig. 45: Bee venom from *Apis mellifera*; Fig. 46: scorpion venom from *Androctonus australis*, and Fig. 47: snake venom from *Bungarus multicinctus*). It may be seen that in all these cases thioredoxin (*E. coli* or human) specifically reduced components of the venoms. Fig. 48 shows that thioredoxin reduces venom components in a similar way when the reaction was performed in the absence of protease inhibitors.

20 25 Fig. 48 represents the results of the reduction of bee, scorpion and snake venoms by the NADP-Thioredoxin system with and without protease inhibitors (SDS-Polyacrylamide gel mBBr procedure). After 20 min incubation at room temperature with NTS in the presence or absence of any protease inhibitors, the samples were derivatized with mBBr, separated by electrophoresis, and fluorescence was determined as in Figs. 45-47.

Materials

30 Venoms: Been venom from *Apis mellifera*, scorpion venom from *Androctonus australis*, and snake venom from *Bungarus multicinctus* were purchased from Sigma chemical Co. (St. Louis, MO).

35 Protease Inhibitors: Phenylmethylsulfonyl fluoride (PMSF), Leupeptin and Pepstatin were purchased from Sigma Chemical Co. (St. Louis, MO).

Venom Detoxification

40 Detoxification of bee, scorpion and snake venoms is determined by subcutaneous injection into mice. Assays are done in triplicate. Prior to injection, the venom is diluted in phosphate-saline buffer (0.15 M NaCl in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.2) at concentrations ranging up to twice the LD<sub>50</sub> (per g mouse): bee venom from *Apis mellifera*, 2.8 µg; scorpion venom from *Androctonus australis*, 0.32 µg; and snake venom from *Bungarus multicinctus*, 0.16 µg. At 5, 10, 30, 60 minutes and 4, 12 and 24 hr after injection, separate groups of challenged mice are injected (1) intravenously and (2) subcutaneously (multiple local injections around the initial injection site). The thioredoxin is reduced with: (1) *E. coli* NADP-thioredoxin system, using 0.08 µg thioredoxin, 0.07 µg NTR and 25 nmoles NADPH; (2) Thioredoxin reduced by DTT or reduced lipoic acid (0.08 µg *E. coli* or human thioredoxin added to 1 nmole dithiothreitol or 1 nmole of reduced lipoic acid). Concentrations are per µg venom injected into the animal; all solutions are prepared in phosphate-saline buffer.

45 50 The effect of thioredoxin on detoxification is determined by (1) comparing the LD<sub>50</sub> with the control group without thioredoxin and (2) following the extent of the local reaction, as evidenced by necrosis, swelling and general discomfort to the animal.

REDUCTION STUDIES FOR REDUCING SNAKE NEUROTOXINS - MATERIALS AND METHODSToxins

55 Porcine pancreas phospholipase A<sub>2</sub>, erabutoxin b and β-bungarotoxin were purchased from Sigma Chemical Co. (St. Louis, Mo). As the phospholipase A<sub>2</sub> was provided in 3.2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution pH 5.5, the protein was dialysed in

30 mM Tris-HCl buffer, pH 7.9, using a centricon 3 KDa cutoff membrane.  $\alpha$ -Bungarotoxin and  $\alpha$ -bungarotoxin<sup>125</sup>I were a kind gift from Dr. Shalla Verrall.

Reagents and Fine chemicals

5 DL- $\alpha$ -Lipoic acid, L- $\alpha$ -phosphatidylcholine from soybean, NADPH and  $\beta$ -mercaptoethanol were purchased from Sigma Chemical Co. (St Louis, Mo.) and monobromobimane (mBBr, trade name thiolite) from Calbiochem (San Diego, Ca.). Reagents for sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, Ca.).

10 Proteins and Enzymes

15 Thioredoxin and NTR were purified from *E. coli* as is described by Jiao, et al., (1992) *Ag. Food Chem. (in press)*. Thioredoxin *h* was purified from wheat germ (Florencio, F.J., et al. (1988) *Arch Biochem Biophys.* **266**:496-507) and thioredoxins *f* and *m* from spinach leaves (Florencio, F.J., et al., *supra*). Human thioredoxin was a kind gift of Dr. Emanuelle Wollman. NADP-malate dehydrogenase was purified from corn leaves (Jacquot, J.-P., et al. (1981) *Plant Physiol.* **68**:300-304) and glutathione reductase from spinach leaves (Florencio, F.J. et al., *supra*). *E. coli* glutaredoxin was a kind gift of Professor A. Holmgren.

20 SDS-Polyacrylamide Gel Electrophoresis

25 SDS-polyacrylamide gel electrophoresis was performed in 10-20% gradient gels of 1.5 mm thickness that were developed for 3 hr at a constant current of 40 mA. Following electrophoresis, gels were soaked for 2 hr in 12% (w/v) trichloroacetic acid and then transferred to a solution containing 40% methanol and 10% acetic acid for 12 hr to remove excess mBBr. The fluorescence of protein-bound mBBr was determined by placing gels on a light box fitted with an ultraviolet light source (365 nm). Gels were photographed with Polaroid positive/negative Landfilm, type 55, through a yellow Wratten gelatin filter No. 8 (cutoff=460 nm) (exposure time 40 sec. at f/4.5). Gels were stained for protein for 1 hr in solution of 0.125% (w/v) Coomassie blue R-250 in 10% acetic acid and 40% methanol. Gels were destained in this same solution from which Coomassie blue was omitted.

30 Polaroid negatives of fluorescent gels and dry stained gels were scanned with a laser densitometer (Pharmacia-LKB Ultroncan XL). The bands were quantified by evaluating areas or height of the peaks with Gelscan XL software.

EXAMPLE 27

35 Reduction of Toxins and Labeling with mBBr

40 Reactions were carried out with 10  $\mu$ g of target toxin in a final volume of 100  $\mu$ l in 30 mM Tris-HCl buffer, pH 7.9, with 0.8  $\mu$ g thioredoxin, 0.7  $\mu$ g NTR (both from *E. coli*) and 2.5 mM NADPH. When thioredoxin was reduced by DTT, NADPH and NTR were omitted and DTT was added to 0.5 mM. Assays with GSH were performed similarly, but at a final concentration of 1 mM. For reduction by glutaredoxin, the thioredoxin and NTR were replaced by 1  $\mu$ g *E. coli* glutaredoxin, 0.38  $\mu$ g glutathione reductase (partially purified from spinach leaves), 1 mM GSH and 2.5 mM NADPH (the combination of these four components is called NADP/glutaredoxin system). Reduction by the reduced form of lipoic acid, was carried out in a volume of 100  $\mu$ l at two concentrations, 100  $\mu$ M and 200  $\mu$ M, both alone and with 0.8  $\mu$ g of thioredoxin. The mixture was incubated for 2 hr at 37C in the case of erabutoxin b and  $\alpha$ -bungarotoxin, 1 hr at room 45 temperature for  $\beta$ -bungarotoxin and 20 min at room temperature for phospholipase A<sub>2</sub>. After incubation, mBBr was added to 1.5 mM and the reaction continued for 15 min at room temperature. The reaction was stopped and excess mBBr derivatized by adding 10  $\mu$ l of 100 mM  $\beta$ -mercaptoethanol, 5  $\mu$ l of 20 % SDS and 10  $\mu$ l 50 % glycerol. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis.

50 Total toxin reduction was accomplished by boiling samples for 3 min in 2 mM DTT. After cooling, the samples were labeled with mBBr and treated as before, except that all samples were again boiled for 2 min prior to loading in the gel. The extent of the reduction of erabutoxin b by the different reductants described above is shown in Fig. 49. Dithiothreitol (DTT) and the reduced forms of thioredoxin and lipoic acid are dithiol reductants as opposed to monothiol reductants like 2-mercaptoethanol and glutathione. DTT is a synthetically prepared chemical agent, whereas thioredoxin and lipoic acid occur within the cell. Evidence presented above demonstrates that lipoic acid is a more specific reductant than dithiothreitol. Dithiothreitol reduced the toxin partly without thioredoxin (lane 5) whereas reduced lipoic acid did not (lane 8). Fig. 52 shows that the NTS or DTT plus thioredoxin are specific reductants for  $\alpha$ -bungarotoxin and  $\beta$ -bungarotoxin.

EXAMPLE 28NADP-Malate Dehydrogenase Activation

5 The ability of snake toxins to activate chloroplast NADP-malate dehydrogenase was carried out by preincubating 5 µg toxin with a limiting thioredoxin concentration (to restrict activation of the enzyme by the thioredoxin): *E. coli* thioredoxin, 0.25 µg; human, 0.9 µg; wheat, 1.15 µg; spinach *f* and *m*, 0.375 and 0.125 µg, respectively. Purified corn NADP-malate dehydrogenase, 1.4 µg, was added to a solution containing 100 mM Tris-HCl, pH 7.9, thioredoxin as indicated, and 10 mM DTT (final volume 0.2 ml). After 25 min, 160 µl of the preincubation mixture was injected into a 1 cm cuvette of 1 ml capacity containing (in 0.79 ml) 100 mM Tris HCl, pH 7.9, and 0.25 mM NADPH. The reaction was started by the addition of 50 µl of 50 mM oxalacetic acid. NADPH oxidation was followed by monitoring the change in absorbance at 340 nm with a Beckman spectrophotometer fitted with a four-way channel changer. Fig. 50 which represents the results of this experiment shows that the reduction by different reduced thioredoxins of erabutoxin b significantly alters the toxin's biological ability to activate NADP-malate dehydrogenase. The results demonstrate that, although there are 10 differences in effectiveness, all thioredoxins tested function to some extent in limiting the effect of the toxin.

15

EXAMPLE 29Proteolysis assay of Erabutoxin b

20 Erabutoxin b, 10 µg was incubated for 2 hr at 37C with 30 mM Tris-HCl buffer pH 7.9 (total volume, 100 µl). As indicated, the buffer was supplemented with 0.8 µg thioredoxin, 0.7 µg NTR and 2.5 mM NADPH. When thioredoxin was reduced by DTT the NTR and NADPH were omitted and DTT was added to 0.5 mM. Following incubation, samples 25 were digested with 0.4 and 2 µg of trypsin for 10 min at 37 C. DTT, 4.8 µl of 50 mM solution, 5 µl of 20% SDS and 10 µl of 50% glycerol were added, samples were boiled for 3 min, and then subjected to SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue and the protein bands quantified by densitometric scanning as described above. The results of the assay are shown in Table XVI below.

30 These results show that reduction of a snake neurotoxin (erabutoxin b) renders the toxin more susceptible to proteolysis. An extension of this conclusion would indicate that administration of reduced thioredoxin as a toxin antidote should help to destroy the toxin owing to the increase in proteolytic inactivation by proteases of the venom.

TABLE XVI

Susceptibility of the Oxidized and Reduced Forms of Erabutoxin b to Trypsin		
Treatment	% Erabutoxin b digested	
	0.4 µg trypsin	2 µg trypsin
Control	0.0	34.1
Reduced, NTS	21.1	57.8
Reduced, DTT	3.1	40.6
Reduced, DTT + Trx	28.0	71.8

45 Erabutoxin b, 10 µg was preincubated for 2 hours at 37C in 30 mM Tris-HCl buffer, pH 7.9, as follows: control, no addition; reduced by *E. coli* NADP/thioredoxin system (NTS), thioredoxin, NTR and NADPH; reduced by DTT, DTT; and reduced by DTT plus thioredoxin, DTT supplemented with *E. coli* thioredoxin. After preincubation 0.4 µg and 2 µg of trypsin were added to the indicated which then were analyzed by SDS-polyacrylamide gel electrophoresis.

EXAMPLE 30Phospholipase A<sub>2</sub> Assay

50 Activity of the oxidized and reduced forms of the phospholipase A<sub>2</sub> component of  $\beta$ -bungarotoxin was determined spectrophotometrically following change in acidity as described by Lobo de Araujo, et al. (1987) *Toxicon* 25:1181-1188. For reduction experiments, 10 µg toxin was incubated in 30 mM Tris-HCl buffer, pH 7.9, containing 0.8 µg thioredoxin, 0.7 µg NTR and 7 mM NADPH (final volume, 35 µl). After 1 hr incubation at room temperature, 20 µl of the reaction mixture was added to a 1 cm cuvette containing 1 ml of assay solution (adjusted to pH 7.6) that contained 10 mM CaCl<sub>2</sub>.

100 mM NaCl, 4 mM sodium cholate, 175  $\mu$ M soybean phosphatidylcholine and 55  $\mu$ M phenol red. The reaction was followed by measuring the change in the absorbance at 558 nm in a Beckman Du model 2400 spectrophotometer. The results of this experiment which are shown in Figure 51, demonstrate that  $\beta$ -bungarotoxin loses most of its phospholipase activity when reduced by thioredoxin. The results are consistent with the conclusion that the administration of 5 reduced thioredoxin following a snake bite would help detoxify the toxin by eliminating phospholipase A<sub>2</sub> activity.

### EXAMPLE 31

#### $\alpha$ -Bungarotoxin binding to Acetylcholine Receptor

10  $\alpha$ -Bungarotoxin binding was assayed with cultured mouse cells by using radiolabeled toxin (Gu, Y., et al. (1985) *J. Neurosci.* 5:1909-1916). Mouse cells, line C<sub>2</sub>, were grown as described by Gu et al (Gu, Y. et al., *supra.*) and plated in 24-well plastic tissue culture plates (Falcon) at a density of about 3000 cells per well. Growth medium was replaced by 15 fusion medium after 48 hr and again after 96 hr. Cultures were used for assay after an additional 2 days growth.

15  $\alpha$ -Bungarotoxin binding was determined with cells subjected to three different treatments: [A] 10 nM  $\alpha$ -bungarotoxin<sup>125</sup>I (262 Ci/mmole) was preincubated 2 hr at 37°C in 200  $\mu$ l of phosphate-saline buffer (0.15M NaCl in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.2) with 4  $\mu$ g thioredoxin, 3.5  $\mu$ g NTR (both from *E. coli*) and 6.25 mM NADPH. In certain 20 cases, the NTR and NADPH were replaced by 1.25 mM DTT. After 2 hr incubation, the mixture was transferred to a well containing mouse cells, washed two times with phosphate-saline, and incubated for 2 hours at 37°C. [B] After washing 25 the cells two times with phosphate-saline buffer, 10 nM  $\alpha$ -bungarotoxin<sup>125</sup>I (in 200  $\mu$ l of phosphate-saline) was added per well. Following a 2 hr incubation at 37°C, cells were washed again with phosphate-saline buffer to remove unbound toxin. As indicated, 200  $\mu$ l saline, supplemented with 0.68 mM CaCl<sub>2</sub>, 0.49 mM MgCl<sub>2</sub>, 4  $\mu$ g thioredoxin, 3.5  $\mu$ g NTR and 30 6.25 mM NADPH were added to the well. The plate was incubated 2 hr at 37°C. NTR and NADPH were omitted from treatment with DTT which was added at 1.25 mM. [C] After washing cells twice with phosphate-saline buffer, 200  $\mu$ l of a solution containing 4  $\mu$ g thioredoxin, 3.5  $\mu$ g NTR and 6.25 mM NADPH, were added to each well. In some cases, 35 NTR and NADPH were replaced with 1.25 mM DTT. The plate was incubated for 2 hr at 37°C. Cells were then washed twice with phosphate-saline buffer to remove the added reductant. Phosphate-saline buffer, 200  $\mu$ l, containing 0.68 mM CaCl<sub>2</sub> and 0.49 mM MgCl<sub>2</sub> and 10 nM  $\alpha$ -bungarotoxin<sup>125</sup>I was added to each well. Incubation was continued for 2 hr at 37°C. The results of this assay are shown in Table XVII. This experiment shows that when reduced by thioredoxin,  $\beta$ -bungarotoxin can no longer bind to the acetylcholine receptor. When extended to the whole animal, the thioredoxin-linked reduction mechanism would result in detoxification by eliminating binding of the toxin to its target receptor.

Each  $\alpha$ -bungarotoxin binding assay was done in triplicate. Nonspecific binding was measured by adding 100-fold excess unlabeled  $\alpha$ -bungarotoxin to the incubation mixture. After the incubation period, the cells in all cases were washed with phosphate-saline to remove unbound toxin. The amount of toxin bound was determined by solubilizing the 35 cells in 0.1 M NaOH and measuring radioactivity in a gamma counter.

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TABLE XVII

Binding of  $\alpha$ -Bungarotoxin to the  
Acetylcholine Receptor of Mouse Cells

<u>Treatment A</u>		<u>Binding</u>
Toxin + Reductant	2 hr, 37°C	cells 2 hr, 37°C STOP
No reductant		100.0
NTS		0.0
DTT plus Thioredoxin		0.0
NTS minus NTR		63.0
NTS minus Thioredoxin		78.0
NTS minus NADPH		101.0
<u>Treatment B</u>		
Toxin + Cells	2 hr, 37°C	wash cells + reductant 2 hr, 37°C STOP
No reductant		100.0
NTS		78.0
DTT plus Thioredoxin		76.0
<u>Treatment C</u>		
Cells + Reductant	2 hr, 37°C	wash cells + toxin 2 hr, 37°C STOP
No reductant		100.0
NTS		68.7
DTT		85.0
DTT plus Thioredoxin		68.8

*E. coli* NTS:thioredoxin, NTR and NADPH

## EXAMPLE 32

Example for Detoxification in an Animal

Detoxification of snake neurotoxins is determined by subcutaneous injection into mice. Assays are done in triplicate. Prior to injection, the toxin is diluted in phosphate-saline buffer (0.15M NaCl in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 7.2) at concentrations ranging up to twice the LD<sub>50</sub> dose. (LD<sub>50</sub> is defined as that dose of toxin that kills 50% of a given group of animals.) For toxicity tests, the following neurotoxin concentrations correspond to the LD<sub>50</sub> (per g mouse): erabutoxin b, 0.05  $\mu$ g - 0.15  $\mu$ g;  $\alpha$ -bungarotoxin, 0.3  $\mu$ g; and  $\beta$ -bungarotoxin, 0.089  $\mu$ g. At 5, 10, 30, 60 minutes and 4, 12 and 24

hr after injection, separate groups of the challenged mice are injected (1) intravenously, and (2) subcutaneously (multiple local injections around the initial injection site). The thioredoxin is reduced with: (1) the *E. coli* NADP-thioredoxin system, using 0.08 µg thioredoxin, 0.07 µg NTR and 25 nanomoles NADPH; (2) Thioredoxin plus 1-2 nanomoles of reduced lipoic acid, using 0.08 µg *E. coli* or 0.20 µg human thioredoxin, and (3) using 0.08 µg *E. coli* or 0.20 µg human thioredoxin with 5 nanomoles dithiothreitol (concentrations are per µg toxin injected into the animal; all solutions are prepared in phosphate-saline buffer).

The effect of thioredoxin on detoxification is determined by (1) comparing the LD<sub>50</sub> with the control group without thioredoxin; (2) following the extent of the local reaction, as evidenced by necrosis, swelling and general discomfort to the animal; (3) following the serum levels of creatin kinase, an indicator of tissue damage. Creatin kinase, which is released into the blood as a result of breakage of muscle cells, is monitored using the standard assay kit obtained from Sigma Chemical Co. (St. Louis, MO).

The symptoms of snake bite are multiple and depend on a variety of factors. As a consequence, they vary from patient to patient. There are, nonetheless, common symptoms that thioredoxin treatment should alleviate in humans. Specifically, the thioredoxin treatment should alleviate symptoms associated with neurotoxic and related effects resulting from snake bite. Included are a decrease in swelling and edema, pain and blistering surrounding the bite; restoration of normal pulse rate; restriction of necrosis in the bite area; minimization of the affected part. A minimization of these symptoms should in turn result in improvement in the general health and state of the patient.

#### CONCLUDING REMARKS

It can be seen from the foregoing general description of the invention and from the specific examples illustrating applications thereof, that the invention has manifold and far reaching consequences. The invention basically provides novel dough and dough mixtures and novel methods for creating new doughs and for improving the quality of dough and baked goods as well as novel methods for inactivating enzyme inhibitors in cereal products. The invention also provides a novel method for altering the biological activity and inactivity of animal toxins, namely bee, scorpion and snake toxins. The invention further provides a novel protein that is a pullulanase inhibitor and a method for its inactivation.

While the invention has described in connection with certain specific embodiments thereof, it should be realized that various modifications as may be apparent to those of skill in the art to which the invention pertains also fall within the scope of the invention as defined by the appended claims.

#### Claims

1. A composition comprising a grain food product containing an added amount of thioredoxin wherein the concentration of the added amount of thioredoxin in the grain food product is at least about .02 ppm of said product.
2. A method of increasing the strength and volume of a dough or a baked good comprising the steps of:
  - (a) mixing a thiol redox protein selected from the group consisting of thioredoxin and glutaredoxin with dough ingredients containing glutenins or gliadins to form a dough, and
  - (b) baking the dough to form a baked good.
3. The method of Claim 2 wherein the thiol redox protein is thioredoxin.
4. The method according to one of the claims 2 and 3 wherein thioredoxin comprises from about 0.01 to about 0.3 ppm of said dough.
5. A method of increasing the strength and volume of a semolina dough or volume of a cooked pasta comprising the steps of:
  - (a) mixing a thiol redox protein selected from the group consisting of thioredoxin and glutaredoxin with semolina dough ingredients containing glutenins or gliadins to form a dough;
  - (b) shaping the dough mixture from step (a), and
  - (c) cooking the shaped dough mixture from step (b) to form a cooked pasta.
6. The method of Claim 5 wherein the thiol redox protein is thioredoxin.
7. A method of producing a dough from rice, corn, soybean, barley, oat, sorghum, cassava or millet flour, comprising

(a) mixing a thiol redox protein selected from the group consisting of thioredoxin and glutaredoxin with said flour to form a mixture, said flour containing storage proteins;  
5 (b) reducing said thiol redox protein in said mixture;  
(c) reducing said storage proteins by said reduced thiol redox protein, and  
(d) oxidizing said reduced storage proteins, said oxidized storage proteins creating a protein network complex in the form of a pliable dough.

8. A method of producing a dough from rice, corn, soybean, barley, oat, sorghum or millet flour, comprising

10 (a) mixing a reduced thiol redox protein selected from the group consisting of thioredoxin and glutaredoxin with said flour and a liquid to form a mixture, said flour containing water insoluble storage proteins;  
(b) reducing said storage proteins by said reduced thiol redox protein, and  
(c) oxidizing said reduced storage proteins, said oxidized storage proteins creating a protein network complex in the form of a pliable dough.

15 9. A dough mixture comprising an added amount of thioredoxin or glutaredoxin, said added thioredoxin or glutaredoxin being from about 0.1 ppm to about 1.0 ppm of said mixture.

10. A method for increasing the strength and volume of a dough comprising

20 (a) mixing lysed yeast cells having expressed thioredoxin, and lysed yeast cells having expressed NADP-thioredoxin reductase, with NADPH or an NADPH generating system and water or a liquid to form a mixture, and  
25 (b) adding said mixture to flour to form a dough, said thioredoxin from said yeast cells comprising from about 0.01 ppm to about 1.0 ppm of said dough.

11. A method for increasing the strength and volume of a dough comprising

30 (a) mixing lysed yeast cells, having expressed thioredoxin, and lysed yeast cells having expressed NADP-thioredoxin reductase, with NADPH or an NADPH generating system to form a mixture, and  
(b) adding said mixture to dough ingredients to form a dough, said thioredoxin from said yeast cells comprising from about 0.01 ppm to about 1.0 ppm of said dough.

12. A method for increasing the strength and volume of a dough comprising

35 (a) mixing yeast cells which express thioredoxin, and yeast cells, which express NADP-thioredoxin reductase, with NADPH or an NADPH generating system and water or a liquid to form a mixture, and  
(b) adding said mixture to flour to form a dough, said thioredoxin from said yeast, cells comprising from about 0.01 ppm to about 1.0 ppm of said dough.

40 13. A method for increasing the volume of a baked good comprising

45 (a) mixing lysed yeast cells having expressed thioredoxin, and lysed yeast cells having expressed NADP-thioredoxin reductase, with NADPH or an NADPH generating system and water or a liquid to form a mixture;  
(b) adding said mixture to flour to form a dough, said thioredoxin from said yeast cells comprising from about 0.01 ppm to about 1.0 ppm of said dough; and  
(c) baking said dough to produce a baked good.

50 14. A method for increasing the volume of a baked good comprising

55 (a) mixing lysed yeast cells having expressed thioredoxin and lysed yeast cells having expressed NADP-thioredoxin reductase with NADPH or an NADPH generating system to form a mixture;  
(b) adding said mixture to dough ingredients to form a dough, said thioredoxin from said yeast cells comprising from about 0.01 ppm to about 1.0 ppm of said dough, and  
(c) baking said dough to produce a baked good.

15. A flour mixture suitable for use in preparing a baked good comprising flour and at least 20 µg of yeast thioredoxin.

16. A method of increasing the volume of a cooked pasta comprising the steps of:

(a) mixing a thiol redox protein selected from the group consisting of thioredoxin and glutaredoxin with pasta dough ingredients to form a dough;

5 (b) shaping the dough mixture from step (a), and

(c) cooking the shaped dough mixture from step (b) to form a cooked pasta.

17. A method of increasing the strength of a dough and the volume of a baked good comprising the steps of:

10 (a) mixing NADPH or an NADPH generating system with dough ingredients containing glutenins or gliadins to form a dough, and

(b) baking the dough to form a baked good.

15 18. A method of increasing the volume of a baked good comprising the steps of:

(a) mixing a thiol redox protein selected from the group consisting of thioredoxin and glutaredoxin with dough ingredients to form a dough;

20 (b) shaping the dough mixture from step (a), and

(c) baking the shaped dough mixture from step (b) to form a baked good.

25 19. A method of increasing the volume of a triticale baked good comprising the steps of:

(a) mixing a liquid and thioredoxin with triticale flour to form a dough mixture, said thioredoxin being in combination with NTR and an NADPH generating system; and

25 (b) baking the dough to form a baked good.

30 20. A baked good containing an added amount of thioredoxin wherein the concentration of the added amount of thioredoxin in the baked good is at least of about 0.02ppm of said baked good.

#### 35 Patentansprüche

1. Zusammensetzung, umfassend ein Nahrungsmittel-Produkt aus Getreide, das eine zugefügte Thioredoxin-Menge enthält, worin die Konzentration der zugefügten Thioredoxin-Menge in dem Nahrungsmittel-Produkt aus Getreide wenigstens etwa 0,02 ppm des Produkts beträgt.
- 35 2. Verfahren zur Erhöhung der Festigkeit und des Volumens eines Teigs oder einer gebackenen Ware, umfassend die Stufen:
  - (a) des Vermischens eines Thiolredox-Proteins, das aus der aus Thioredoxin und Glutaredoxin bestehenden Gruppe ausgewählt ist, mit Teig-Bestandteilen, die Glutenine oder Gliadine enthalten, um einen Teig zu bilden, und
  - 40 (b) des Backens des Teigs, um eine gebackene Ware zu bilden.
3. Verfahren gemäß Anspruch 2, worin das Thiolredox-Protein Thioredoxin ist.
- 45 4. Verfahren gemäß einem der Ansprüchen 2 und 3, worin das Thioredoxin etwa 0,01 bis etwa 0,3 ppm des Teigs umfaßt.
5. Verfahren zur Erhöhung der Festigkeit und des Volumens eines Griesmehlteigs oder des Volumens einer gekochten Teigware, umfassend die Stufen:
  - (a) des Vermischens eines Thiolredox-Proteins, das aus der aus Thioredoxin und Glutaredoxin bestehenden Gruppe ausgewählt ist, mit Griesmehlteig-Bestandteilen, die Glutenine oder Gliadine enthalten, um einen Teig zu bilden, und
  - 55 (b) Formens der Teig-Mischung der Stufe (a), und
  - (c) des Kochens der geformten Teig-Mischung der Stufe (b), um eine gekochte Teigware zu bilden.
6. Verfahren gemäß Anspruch 5, worin das Thiolredox-Protein Thioredoxin ist.

7. Verfahren zur Herstellung eines Teigs aus Reis-, Mais-, Sojabohnen-, Gerste-, Hafer-, Sorghum-, Tapioka- oder Hirsemehl, umfassend

5 (a) das Vermischen eines Thiolredox-Proteins, das aus der aus Thioredoxin und Glutaredoxin bestehenden Gruppe ausgewählt ist, mit dem Mehl, um eine Mischung zu bilden, wobei das Mehl Speicherproteine enthält;

(b) die Reduktion des Thiolredox-Proteins in der Mischung;

(c) die Reduktion der Speicherproteine durch das reduzierte Thiolredox-Protein, und

(d) die Oxidation der reduzierten Speicherproteine, wobei die oxidierten Speicherproteine einen Protein-Netzwerkkomplex in Form eines biegsamen Teigs bilden.

10 8. Verfahren zur Herstellung eines Teigs aus Reis-, Mais-, Sojabohnen-, Gerste-, Hafer-, Sorghum- oder Hirsemehl, umfassend

15 (a) das Vermischen eines Thiolredox-Proteins, das aus der aus Thioredoxin und Glutaredoxin bestehenden Gruppe ausgewählt ist, mit dem Mehl und einer Flüssigkeit, um eine Mischung zu bilden, wobei das Mehl wasserunlösliche Speicherproteine enthält;

(b) die Reduktion der Speicherproteine durch das reduzierte Thiolredox-Protein, und

(c) die Oxidation der reduzierten Speicherproteine, wobei die oxidierten Speicherproteine einen Protein-Netzwerkkomplex in Form eines biegsamen Teigs bilden.

20 9. Teig-Mischung, umfassend eine zugefügte Menge von Thioredoxin oder Glutaredoxin, wobei das zugefügte Thioredoxin und das zugefügte Glutaredoxin etwa 0,1 ppm bis etwa 1,0 ppm der Mischung ausmachen.

10. Verfahren zur Erhöhung der Festigkeit und des Volumens eines Teigs, umfassend

25 (a) das Vermischen lysierter Hefezellen, die exprimierte Thioredoxin aufweisen, und lysierter Hefezellen, die exprimierte NADP-Thioredoxin-Reduktase aufweisen, mit NADPH oder einem NADPH-erzeugenden System und Wasser oder einer Flüssigkeit, um eine Mischung zu bilden, und

(b) die Zugabe dieser Mischung zu Mehl, um einen Teig zu bilden, wobei das Thioredoxin aus den Hefezellen etwa 0,01 ppm bis etwa 1,0 ppm des Teigs umfaßt.

30 11. Verfahren zur Erhöhung der Festigkeit und des Volumens eines Teigs, umfassend

35 (a) das Vermischen lysierter Hefezellen, die exprimierte Thioredoxin aufweisen, und lysierter Hefezellen, die exprimierte NADP-Thioredoxin-Reduktase aufweisen, mit NADPH oder einem NADPH-erzeugenden System, um eine Mischung zu bilden, und

(b) die Zugabe der Mischung zu Teig-Bestandteilen, um einen Teig zu bilden, wobei das Thioredoxin aus den Hefezellen etwa 0,01 ppm bis etwa 1,0 ppm des Teigs umfaßt.

40 12. Verfahren zur Erhöhung der Festigkeit und des Volumens eines Teigs, umfassend

(a) das Vermischen von Hefezellen, die Thioredoxin exprimieren, und Hefezellen, die NADP-Thioredoxin-Reduktase exprimieren, mit NADPH oder einem NADPH-erzeugenden System und Wasser oder einer Flüssigkeit, um eine Mischung zu bilden, und

(b) die Zugabe der Mischung zu Mehl, um einen Teig zu bilden, wobei das Thioredoxin aus den Hefezellen etwa 0,01 ppm bis etwa 1,0 ppm des Teigs umfaßt.

45 13. Verfahren zur Erhöhung des Volumens einer gebackenen Ware, umfassend

50 (a) das Vermischen lysierter Hefezellen, die exprimierte Thioredoxin aufweisen, und lysierter Hefezellen, die exprimierte NADP-Thioredoxin-Reduktase aufweisen, mit NADPH oder einem NADPH-erzeugenden System und Wasser oder einer Flüssigkeit, um eine Mischung zu bilden,

(b) die Zugabe der Mischung zu Mehl, um einen Teig zu bilden, wobei das Thioredoxin aus den Hefezellen etwa 0,01 ppm bis etwa 1,0 ppm des Teigs umfaßt, und

55 (c) das Backen des Teigs, um eine gebackene Ware zu erzeugen.

14. Verfahren zur Erhöhung des Volumens einer gebackenen Ware, umfassend

(a) das Vermischen lysierter Hefezellen, die exprimierte Thioredoxin aufweisen, und lysierter Hefezellen, die exprimierte NADP-Thioredoxin-Reduktase aufweisen, mit NADPH oder einem NADPH-erzeugenden System, um eine Mischung zu bilden,

5 (b) die Zugabe der Mischung zu Teig-Bestandteilen, um einen Teig zu bilden, wobei das Thioredoxin aus den Hefezellen etwa 0,01 ppm bis etwa 1,0 ppm des Teigs umfaßt, und

(c) das Backen des Teigs, um eine gebackene Ware zu erzeugen.

15 15. Mehl-Mischung, die geeignet ist, um bei der Herstellung einer gebackenen Ware verwendet zu werden, umfassend Mehl und wenigstens 20 µg Hefe-Thioredoxin.

10 16. Verfahren zur Erhöhung des Volumens einer gekochten Teigware, umfassend die Stufen

(a) das Vermischen eines Thiolredox-Proteins, das aus der aus Thioredoxin und Glutaredoxin bestehenden Gruppe ausgewählt ist, mit Teigwaren-Teig, um einen Teig zu bilden,

15 (b) des Formens der Teig-Mischung der Stufe (a), und

(c) das Kochens der geformten Teig-Mischung der Stufe (b), um eine gekochte Teigware zu bilden.

17. Verfahren zur Erhöhung der Festigkeit eines Teigs und des Volumens einer gebackenen Ware, umfassend die Stufen

20 (a) des Vermischens von NDAPH oder eines NDAPH-erzeugendes Systems mit Teig-Bestandteilen, die Glutenine oder Gliadine enthalten, um einen Teig zu bilden, und

(b) des Backens des Teigs, um eine gebackene Ware zu bilden.

25 18. Verfahren zur Erhöhung des Volumens einer gebackenen Ware, umfassend die Stufen

(a) des Vermischens eines Thiolredox-Proteins, das aus der aus Thioredoxin und Glutaredoxin bestehenden Gruppe ausgewählt ist, mit Teig-Bestandteilen, um einen Teig zu bilden

30 (b) des Formens der Teig-Mischung der Stufe (a), und

(c) des Backens der geformten Teig-Mischung der Stufe (b), um eine gebackene Ware zu bilden.

19. Verfahren zur Erhöhung des Volumens einer gebackenen Triticale-Ware umfassend die Stufen

35 (a) des Vermischens einer Flüssigkeit und Thioredoxin mit Triticale-Mehl, um eine Teig-Mischung zu bilden, wobei das Thioredoxin in Kombination mit NTR und einem NADPH-erzeugenden System vorliegt,

(b) des Backens des Teigs, um eine gebackene Ware zu bilden.

20. Gebackene Ware, die eine zugefügte Thioredoxin-Menge enthält, worin die Konzentration der zugefügten Thioredoxin-Menge in der gebackenen Ware wenigstens etwa 0,02 ppm der gebackenen Ware ausmacht.

40 Revendications

1. Composition comprenant un produit alimentaire à grains qui contient une quantité ajoutée de thiorédoxine, dans laquelle la concentration de la quantité ajoutée de thiorédoxine dans le produit alimentaire à grains est d'au moins environ 0,02 ppm dudit produit.

45 2. Procédé pour accroître la résistance et le volume d'une pâte ou d'un produit cuit comprenant les étapes de:

(a) mélange d'une protéine thiol thiol choisie dans le groupe constitué par la thiorédoxine et la glutarédoxine, et les ingrédients de la pâte contenant des gluténines ou des gliadines pour former une pâte, et

50 (b) cuisson de la pâte pour former un aliment cuit.

3. Procédé selon de la revendication 2 dans lequel la protéine thiol redox est la thiorédoxine.

4. Procédé selon l'une des revendications 2 et 3, dans lequel la thiorédoxine constitue d'environ 0,01 à environ 0,3 ppm de ladite pâte.

55 5. Procédé pour accroître la résistance et le volume d'une pâte de semoule ou le volume d'une pâte cuite, compre-

nant les étapes de :

5 (a) mélange d'une protéine thiol redox choisie dans le groupe constitué par la thiorédoxine et la glutarédoxine, les ingrédients de la pâte de semoule contenant des gluténines ou des gliadines pour former une pâte;

(b) formation du mélange de pâte de l'étape (a), et

(c) cuisson du mélange de pâte formé de l'étape (b) pour former une pâte cuite.

6. Procédé selon la revendication 5, dans lequel la protéine thiol redox est la thiorédoxine.

10 7. Procédé de production d'une pâte à partir de farine de riz, de maïs, de soja, d'orge, d'avoine, de sorgho, de cassave ou de millet, dans lequel

15 (a) on mélange une protéine thiol redox choisie dans le groupe constitué par la thiorédoxine et la glutarédoxine avec ladite farine pour former un mélange, ladite farine contenant des protéines de conservation ;

(b) on réduit ladite protéine thiol redox dans ledit mélange;

(c) on réduit lesdites protéines de conservation avec ladite protéine thiol redox réduite, et

(d) on oxyde lesdites protéines de conservation réduites, lesdites protéines de conservation oxydées créant un complexe de réseaux de protéines sous la forme d'une pâte souple.

20 8. Procédé de production d'une pâte à partir de farine de riz, de maïs, de soja, d'orge, d'avoine, de sorgho ou de millet, dans lequel

25 (a) on mélange une protéine thiol redox réduite choisie dans le groupe constitué par la thiorédoxine et la glutarédoxine avec ladite farine et un liquide pour former un mélange, ladite farine contenant des protéines de conservation insolubles dans l'eau ;

(b) on réduit lesdites protéines de conservation avec ladite protéine thiol redox réduite, et

(c) on oxyde lesdites protéines de conservation réduites, lesdites protéines de conservation oxydées créant un complexe de réseau de protéines sous la forme d'une pâte souple.

30 9. Mélange pour pâte comprenant une quantité ajoutée de thiorédoxine ou de glutarédoxine, ladite thiorédoxine ou glutarédoxine ajoutée représentant d'environ 0,1 ppm à environ 1,0 ppm dudit mélange.

10. Procédé pour accroître la résistance et le volume d'une pâte, dans lequel

35 (a) on mélange des cellules de levure lysées ayant exprimé de la thiorédoxine, et des cellules de levure lysées ayant exprimé de la NADP-thiorédoxine réductase, avec du NADPH ou un système produisant du NADPH et de l'eau ou un liquide pour former un mélange, et

(b) on ajoute ledit mélange à de la farine pour former une pâte, ladite thiorédoxine provenant desdites cellules de levure comprenant d'environ 0,01 ppm à environ 1,0 ppm de ladite pâte.

40 11. Procédé pour accroître la résistance et le volume d'une pâte, dans lequel

45 (a) on mélange des cellules de levure lysées ayant exprimé de la thiorédoxine exprimée, et des cellules de levure lysées ayant exprimé de la NADP-thiorédoxine réductase, avec du NADPH ou un système produisant du NADPH pour former un mélange, et

(b) on ajoute ledit mélange à des ingrédients de pâte pour former une pâte, ladite thiorédoxine provenant desdites cellules de levure constituant d'environ 0,01 ppm à environ 1,0 ppm de ladite pâte.

50 12. Procédé pour accroître la résistance et le volume d'une pâte, dans lequel

55 (a) on mélange des cellules de levure qui expriment de la thiorédoxine et des cellules de levure qui expriment de la NADP-thiorédoxine reductase, avec du NADPH ou un système produisant du NADPH et de l'eau ou un liquide pour former un mélange, et

(b) on ajoute ledit mélange à de la farine pour former une pâte, ladite thiorédoxine provenant desdites cellules de levure constituant d'environ 0,01 ppm à environ 1,0 ppm de ladite pâte.

13. Procédé pour accroître le volume d'un aliment cuit, dans lequel

(a) on mélange des cellules de levure lysées ayant exprimé de la thiorédoxine, et des cellules de levure lysées ayant exprimé de la NADP-thiorédoxine réductase, avec du NADPH ou un système produisant du NADPH et de l'eau ou un liquide pour former un mélange;

5 (b) on ajoute ledit mélange à de la farine pour former une pâte, ladite thiorédoxine provenant desdites cellules de levure constituant d'environ 0,01 ppm à environ 1,0 ppm de ladite pâte; et

(c) on fait cuire ladite pâte pour produire un aliment cuit.

14. Procédé pour accroître le volume d'un aliment cuit, dans lequel

10 (a) on mélange des cellules de levure lysées ayant exprimé de la thiorédoxine et des cellules de levure lysées ayant exprimé de la NADP-thiorédoxine réductase avec du NADPH ou un système produisant du NADPH pour former un mélange;

15 (b) on ajoute ledit mélange à des ingrédients de pâte pour former une pâte, ladite thiorédoxine provenant desdites cellules de levure constituant d'environ 0,01 ppm à environ 1,0 ppm de ladite pâte, et

(c) on fait cuire ladite pâte pour produire un aliment cuit.

15. Mélange de farine approprié pour une utilisation dans la préparation d'un aliment cuit comprenant de la farine et au moins 20 µg de thiorédoxine de levure.

20 16. Procédé pour accroître le volume d'une pâte cuite comprenant les étapes de:

(a) mélange d'une protéine thiol redox choisie dans le groupe constitué par la thiorédoxine et la glutarédoxine avec des ingrédients de pâte pour former une pâte;

25 (b) fromage du mélange de pâte de l'étape (a), et

(c) cuisson du mélange de pâte formé de l'étape (b) pour former une pâte cuite.

17. Procédé pour accroître la résistance d'une pâte et le volume d'un aliment cuit, comprenant les étapes de:

30 (a) mélange de NADPH ou d'un système produisant du NADPH avec des ingrédients de pâte contenant des gluténines ou des gliadines pour former une pâte, et

(b) cuisson de la pâte pour former un aliment cuit.

18. Procédé pour accroître le volume d'un aliment cuit, comprenant les étapes de:

35 (a) mélange d'une protéine thiol redox choisie dans le groupe constitué par la thiorédoxine et la glutarédoxine avec des ingrédients de pâte pour former une pâte;

(b) formation du mélange de pâte de l'étape (a), et

(c) cuisson du mélange de pâte formé de l'étape (b) pour former un aliment cuit.

40 19. Procédé pour accroître le volume d'un aliment cuit à base de blé comprenant les étapes de:

(a) mélange d'un liquide et de thiorédoxine avec de la farine de blé pour former un mélange de pâte, ladite thiorédoxine étant en combinaison avec du NTR et un système produisant du NADPH;

(b) cuisson de la pâte pour former un aliment cuit.

45 20. Article cuit contenant une quantité ajoutée de thiorédoxine, la concentration de la quantité ajoutée de thiorédoxine dans l'aliment cuit étant d'au moins environ 0,02 ppm dudit aliment cuit.

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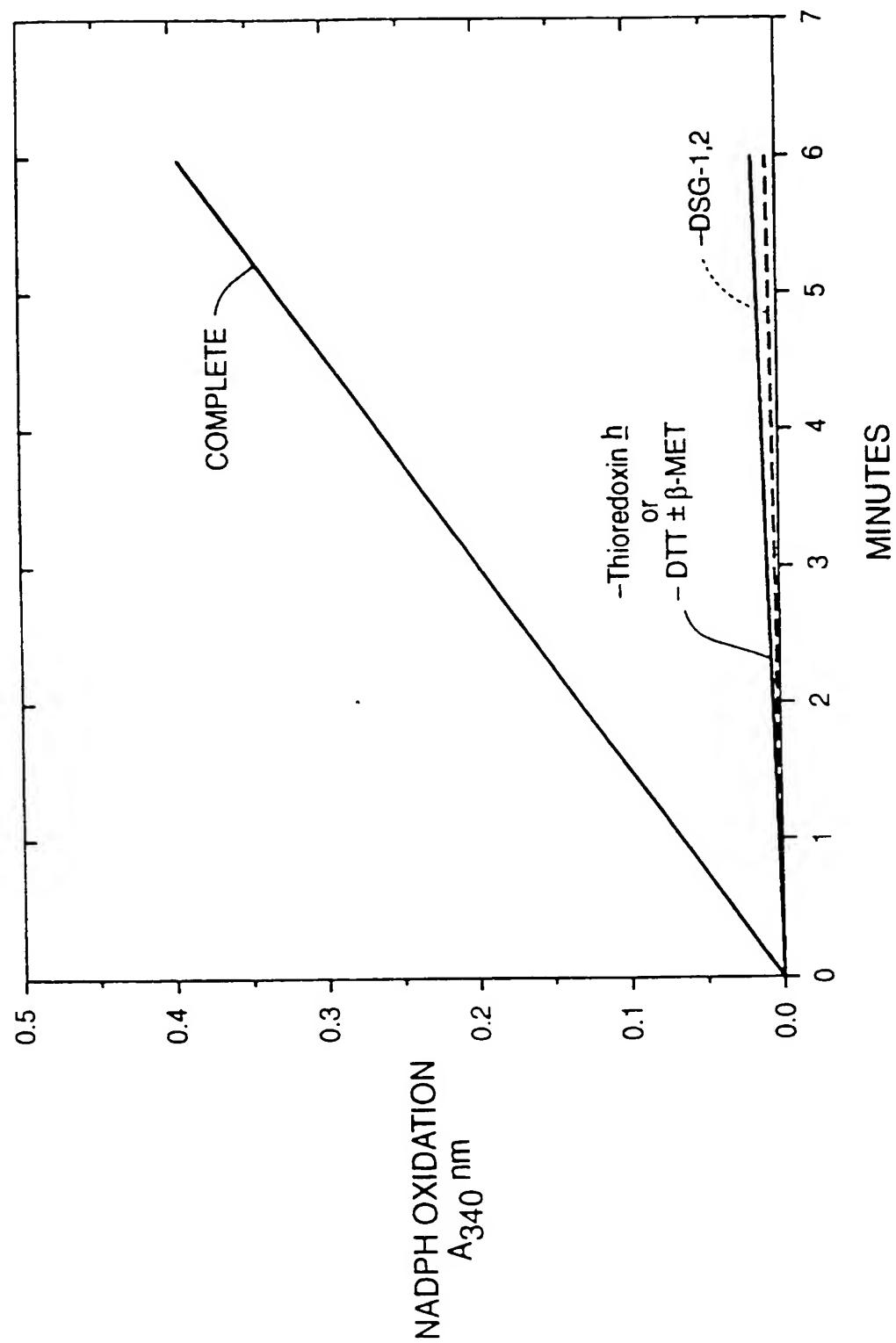


FIG. - 1

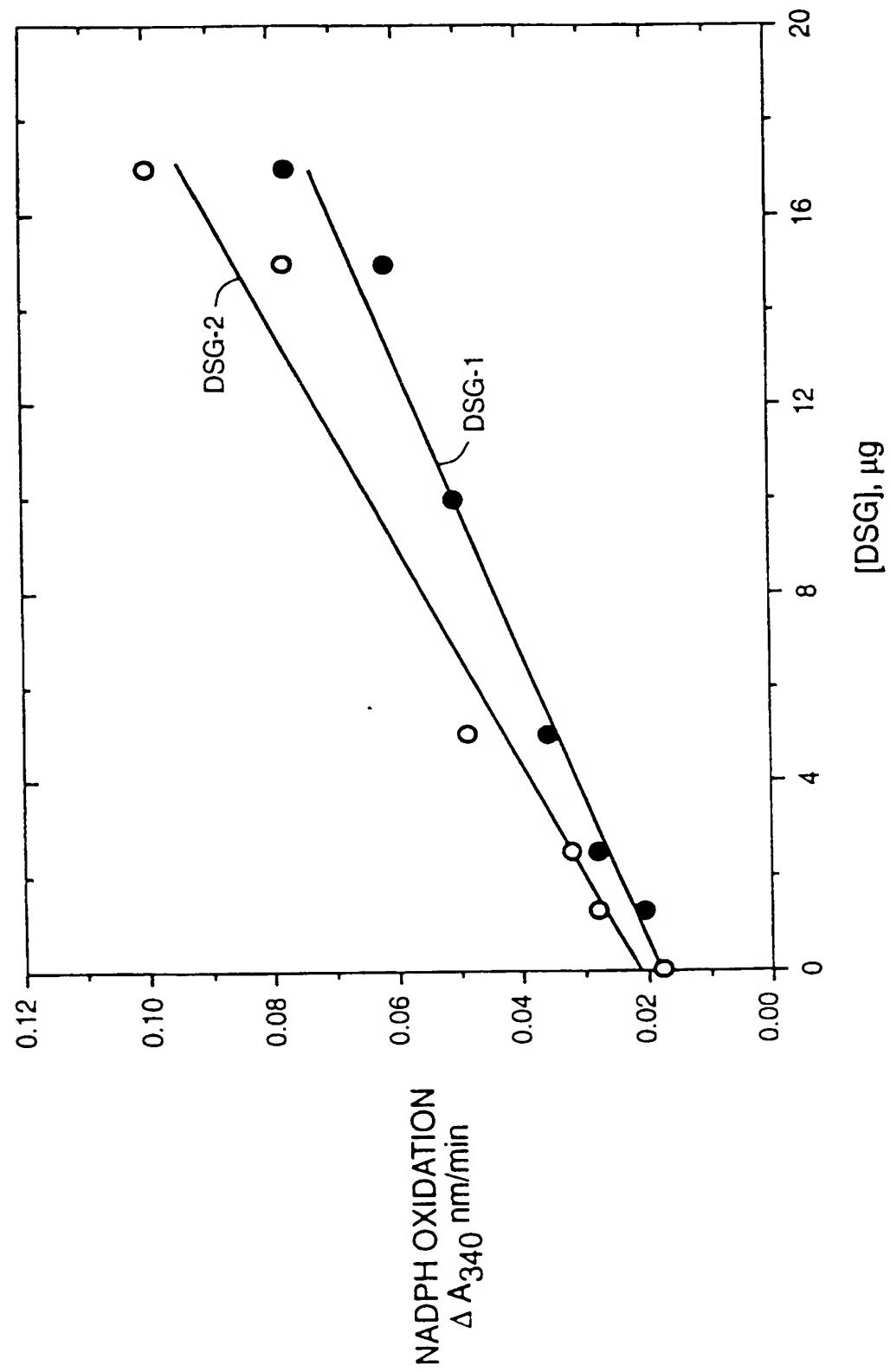


FIG. 2

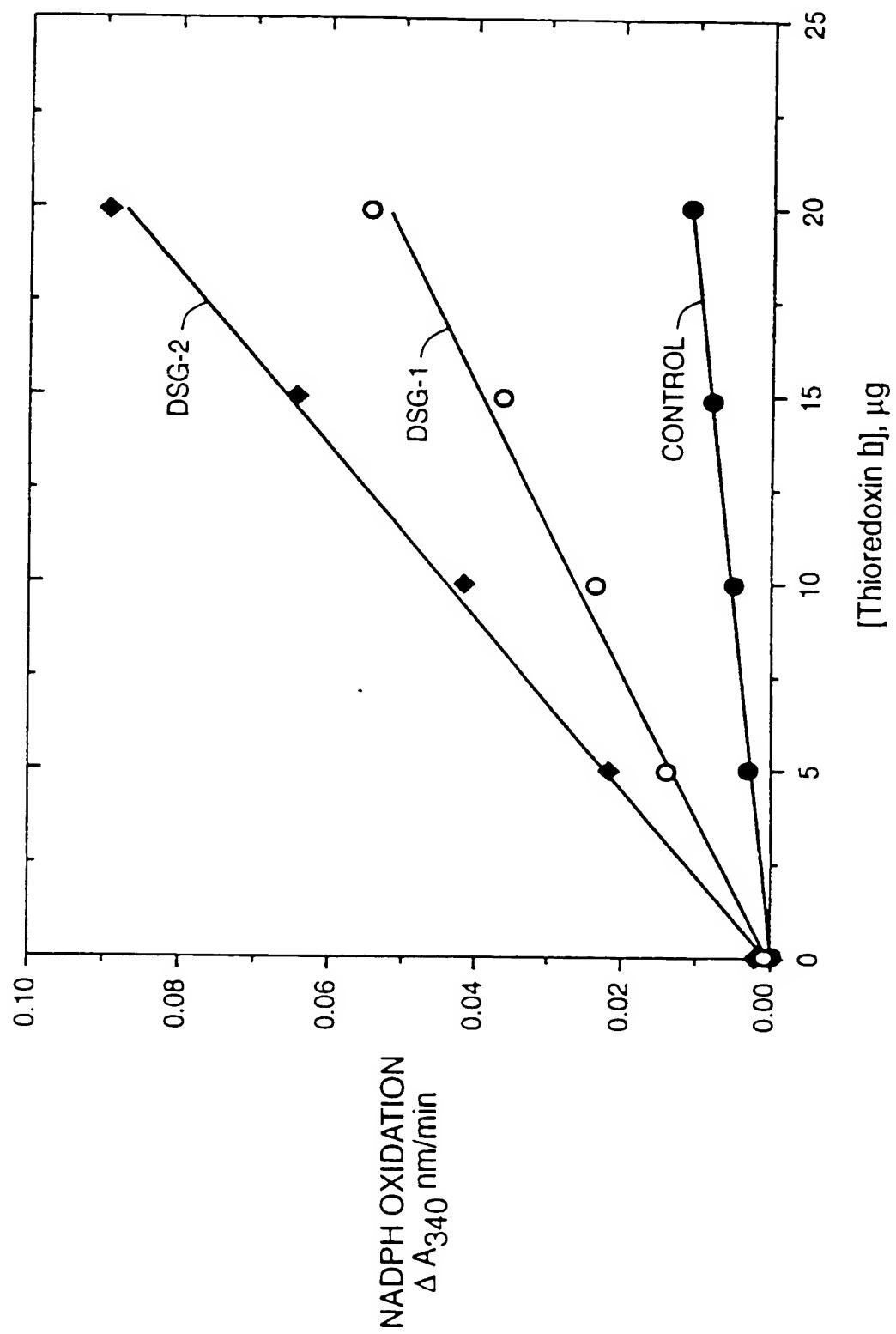


FIG. 3

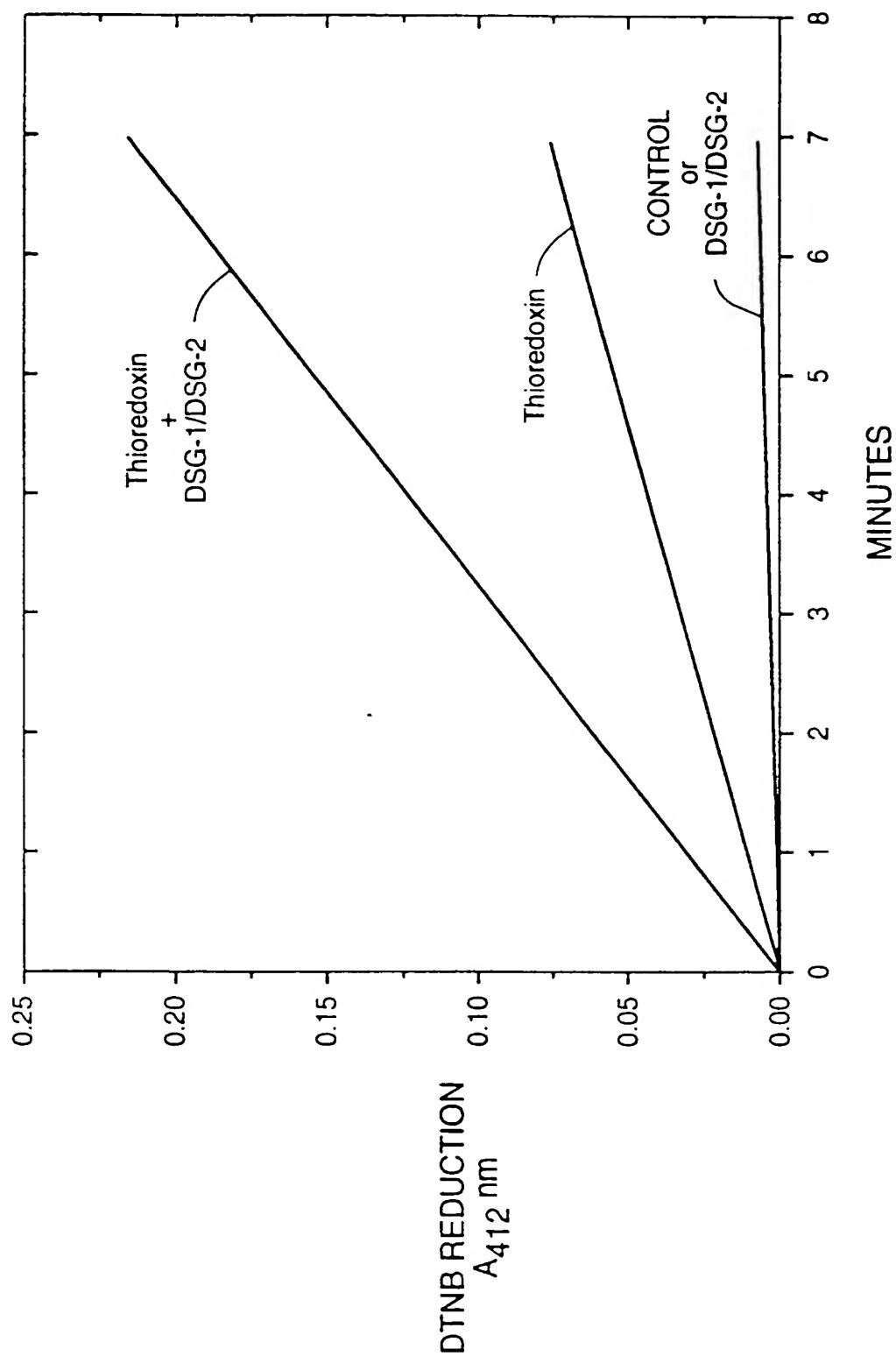


FIG. 4

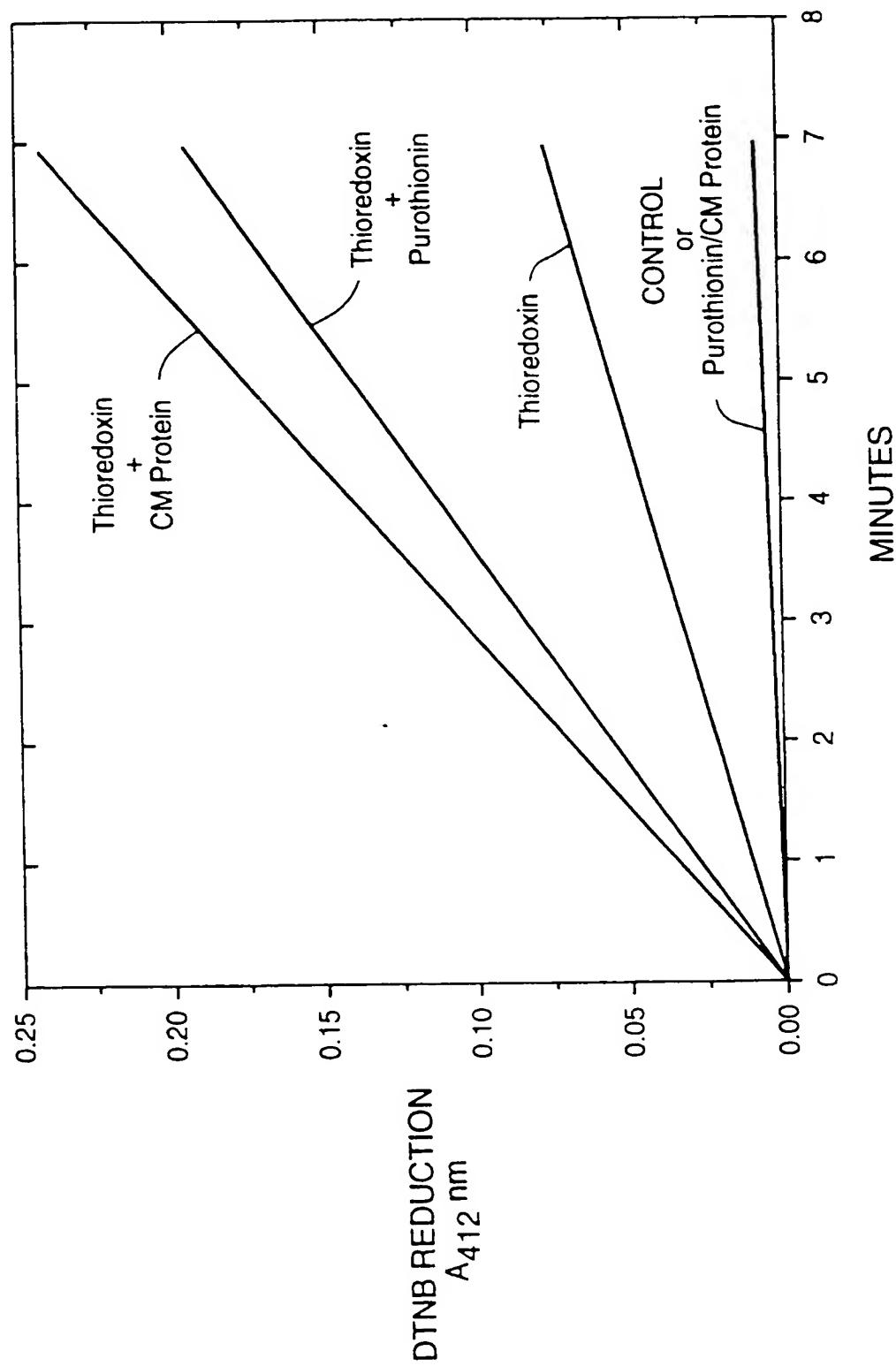
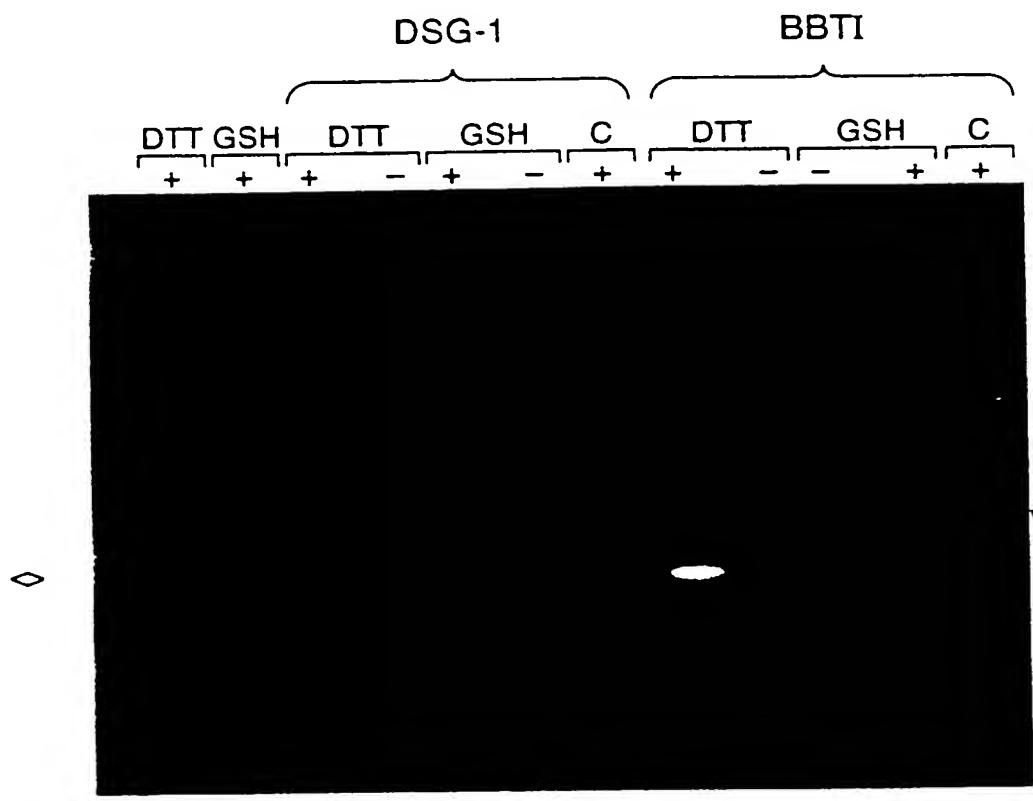


FIG. 5

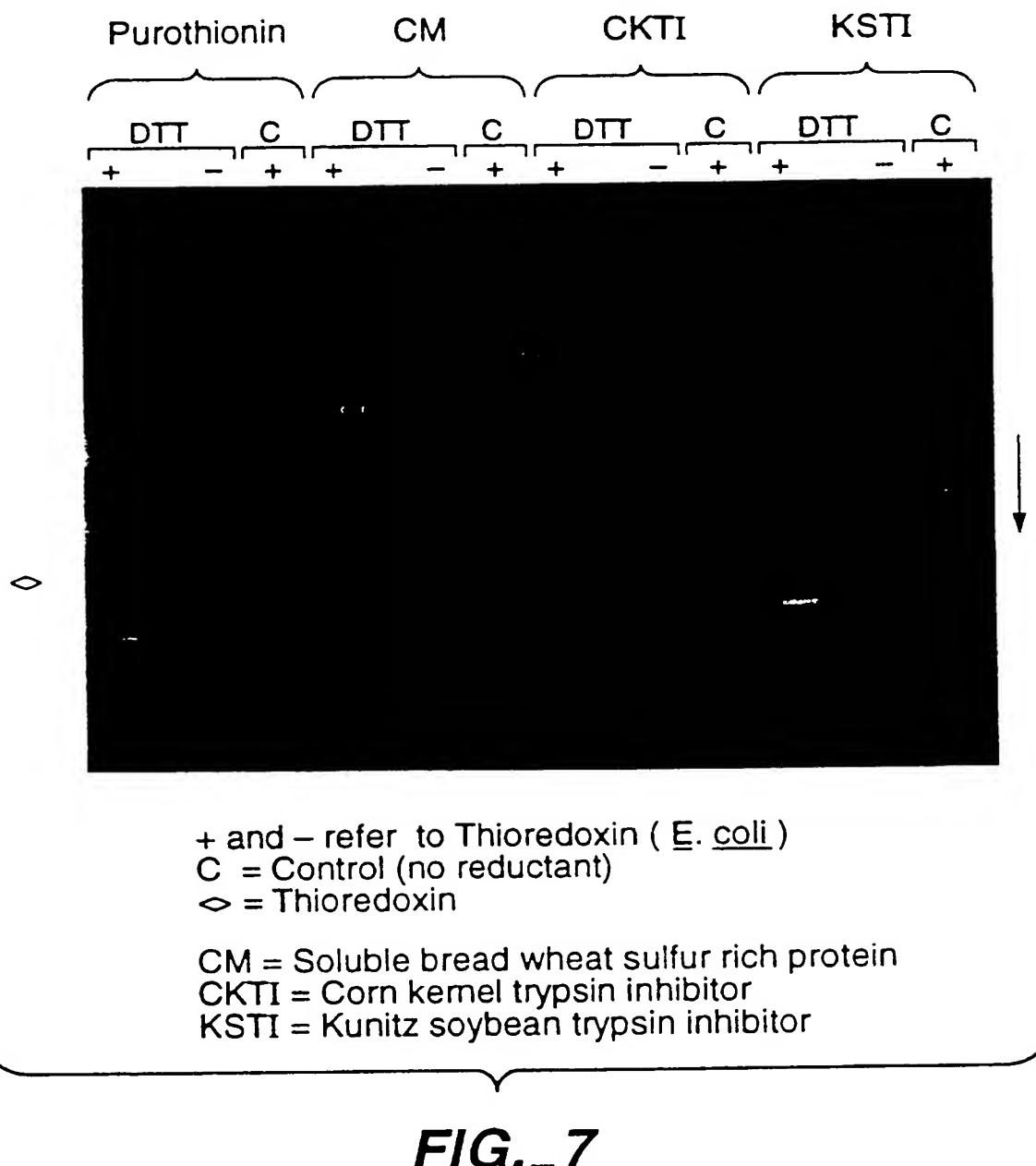


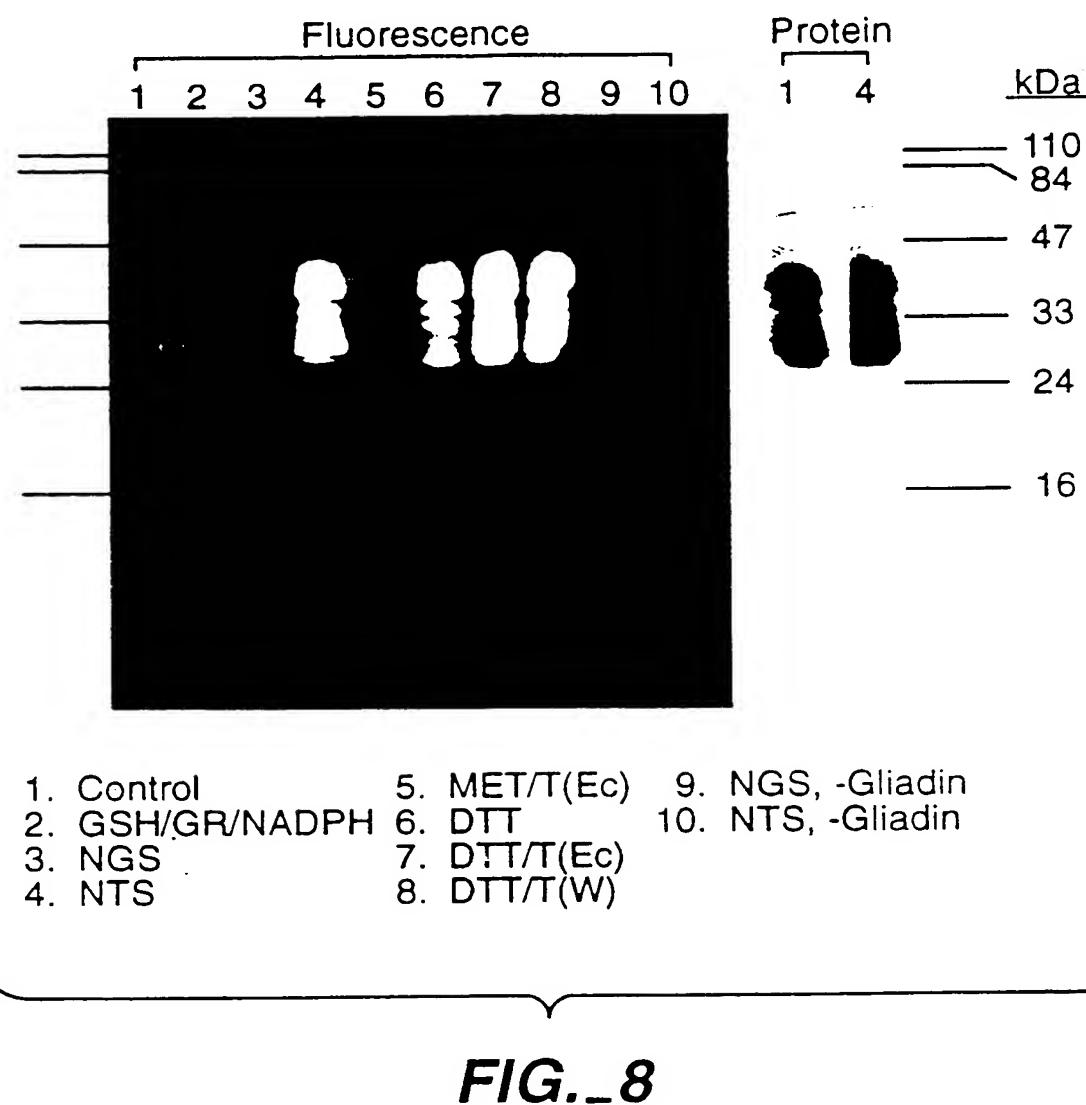
+ and - refer to Thioredoxin ( *E. coli* )

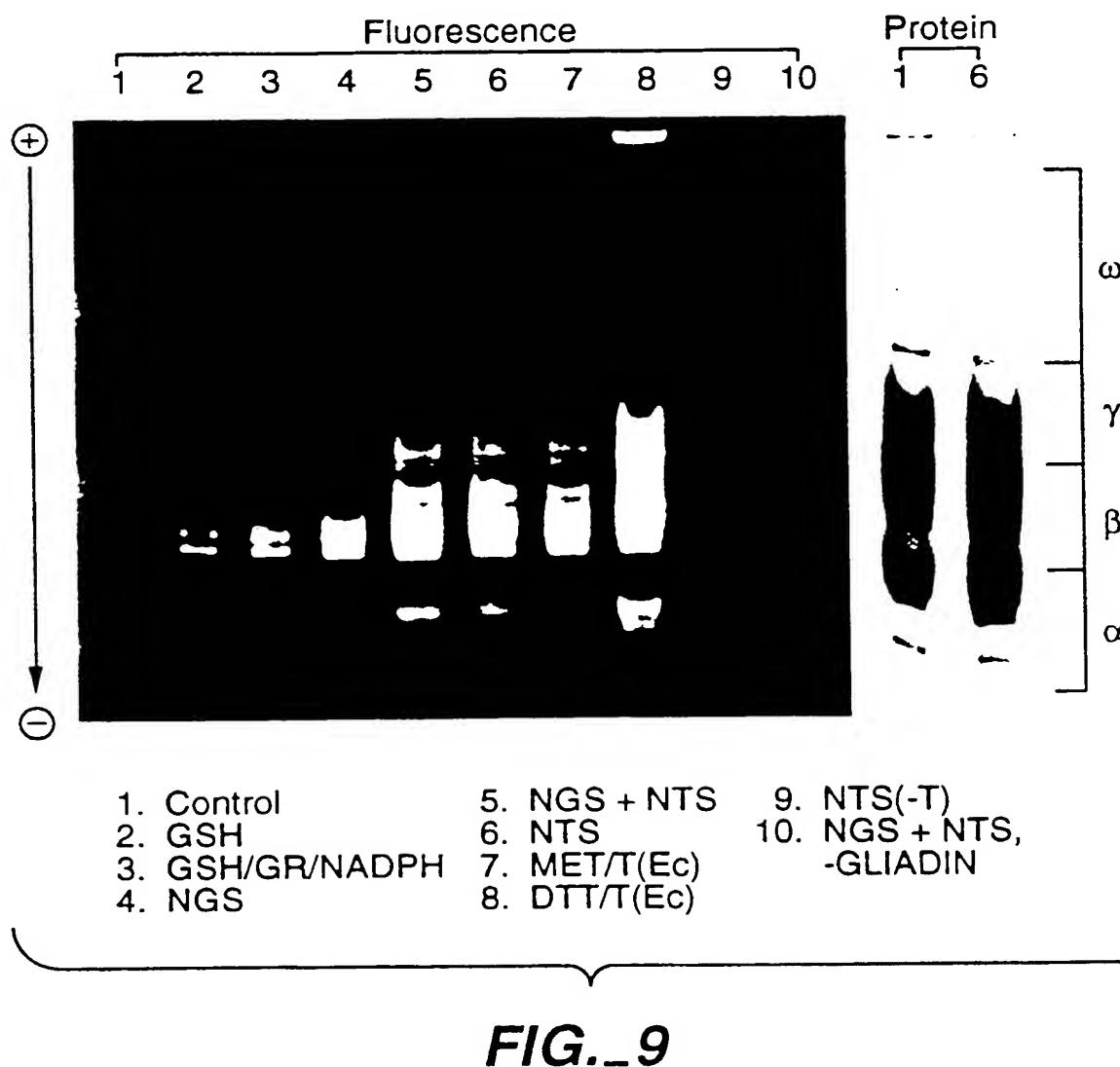
C = Control (no reductant)

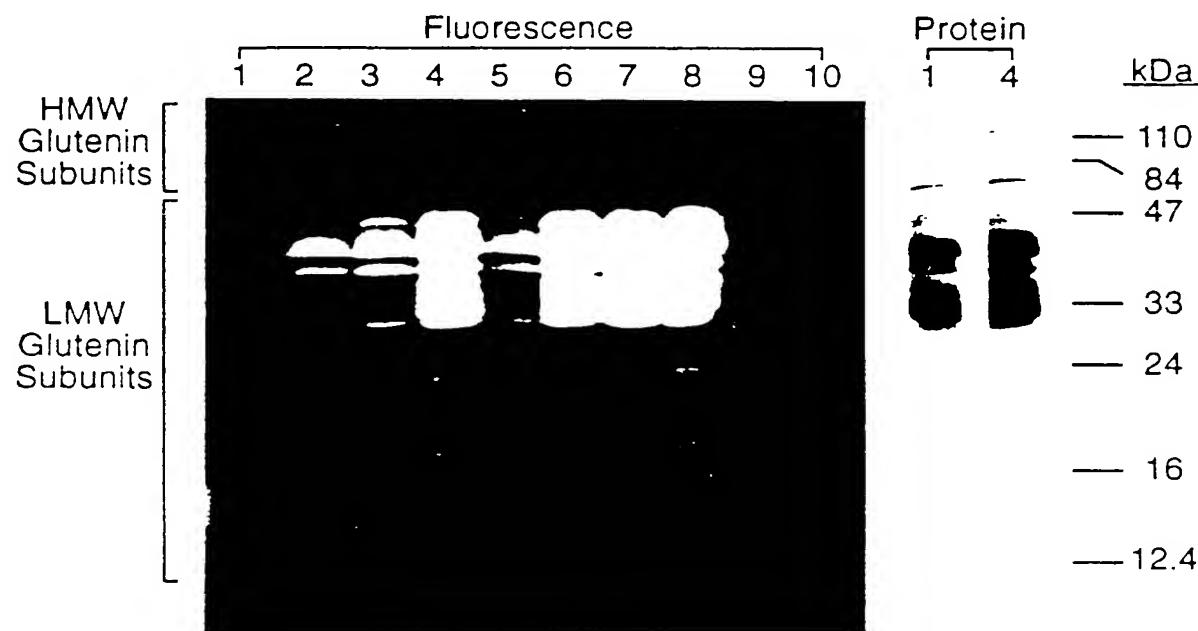
◇ = Thioredoxin

**FIG.\_6**









1. Control
2. GSH/GR/NADPH
3. NGS
4. NTS
5. MET/T(Ec)
6. DTT
7. DTT/T(Ec)
8. DTT/T(W)
9. NGS, -Glutenin
10. NTS, -Glutenin

FIG.\_10

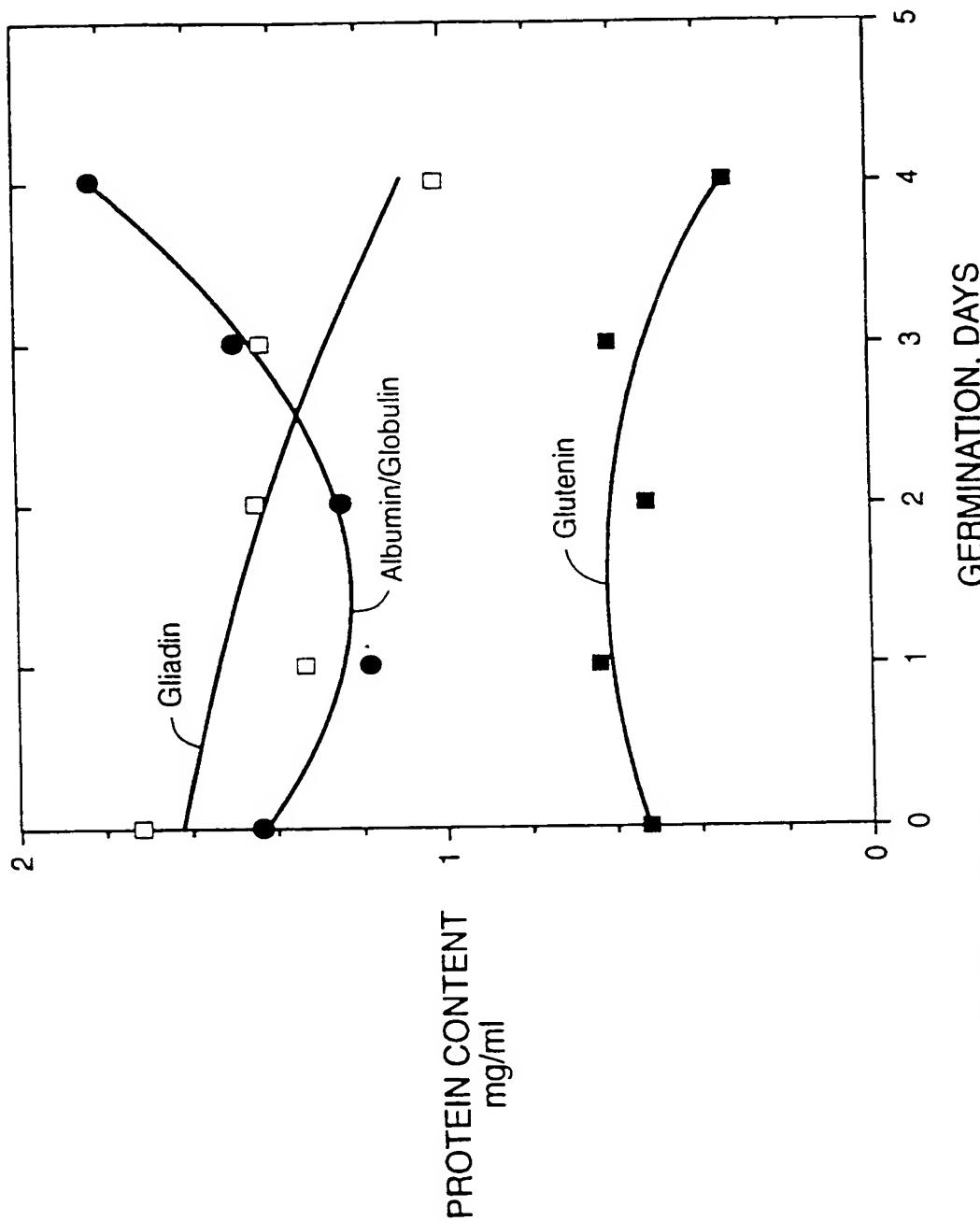


FIG.-11A

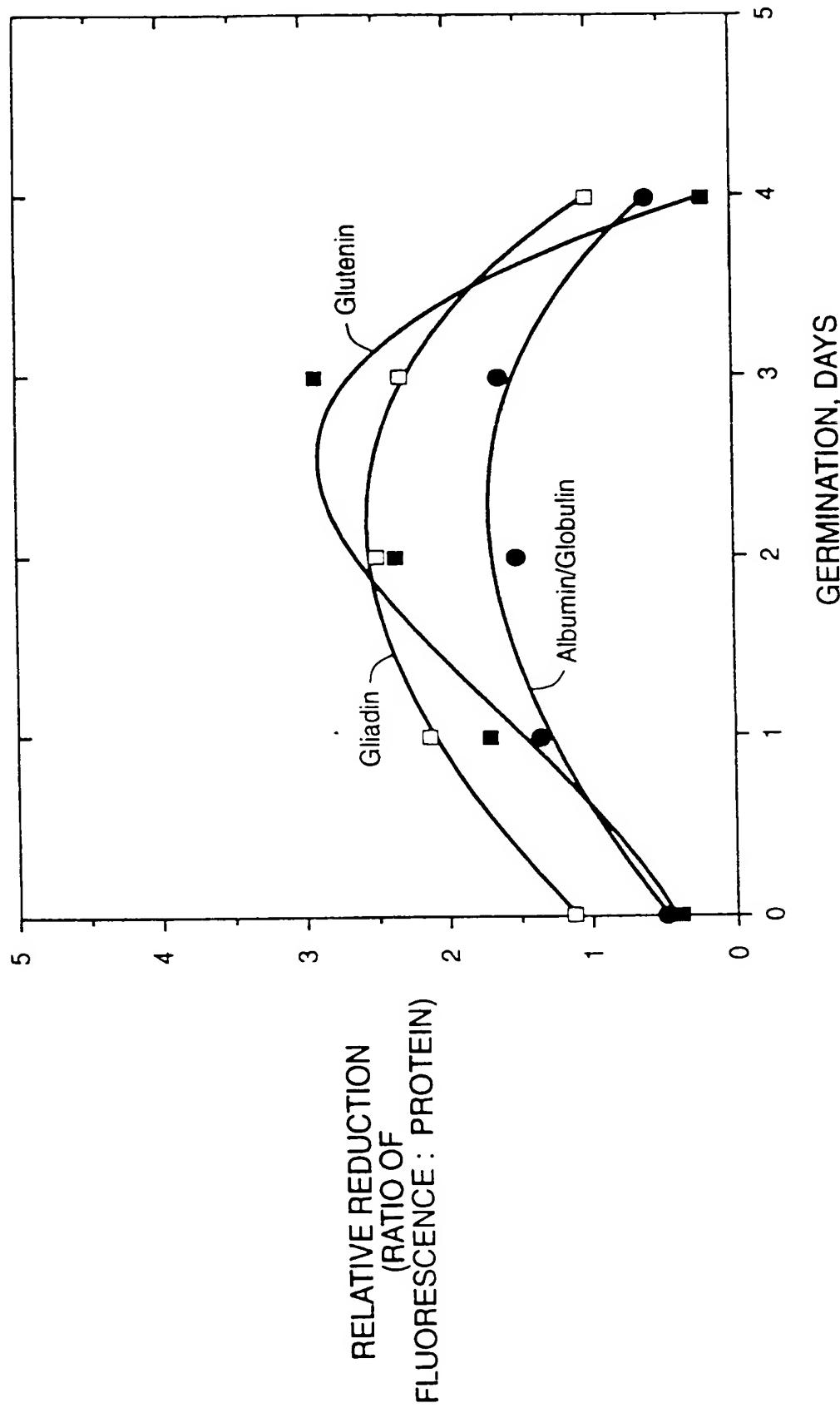


FIG. 11B

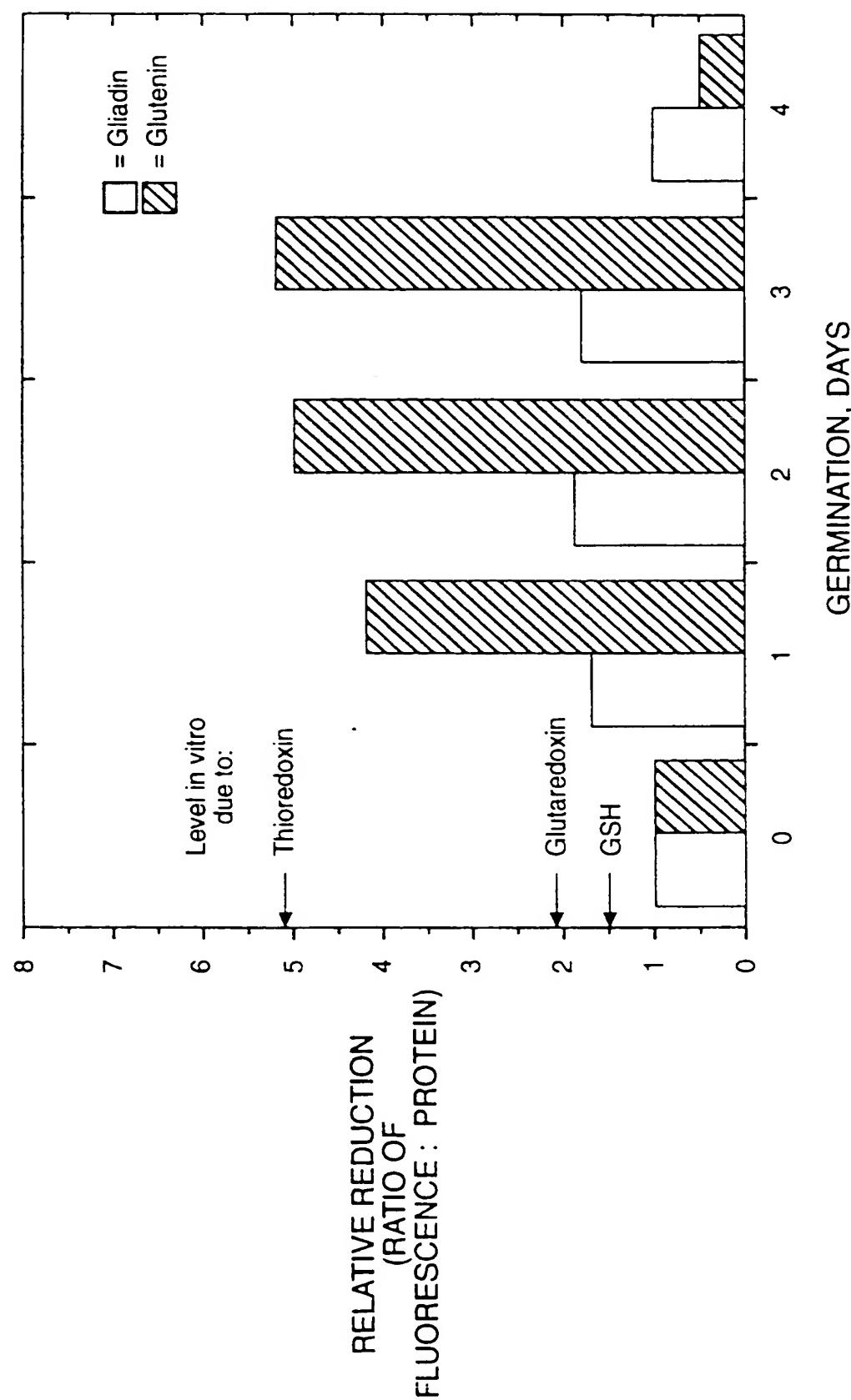


FIG.-12

Flour or Semolina  
[Oxidized Proteins (S-S)]

Dough or Fresh Pasta  
[Reduced Proteins (SH)]

Final Products  
[Interacting Reduced Proteins]

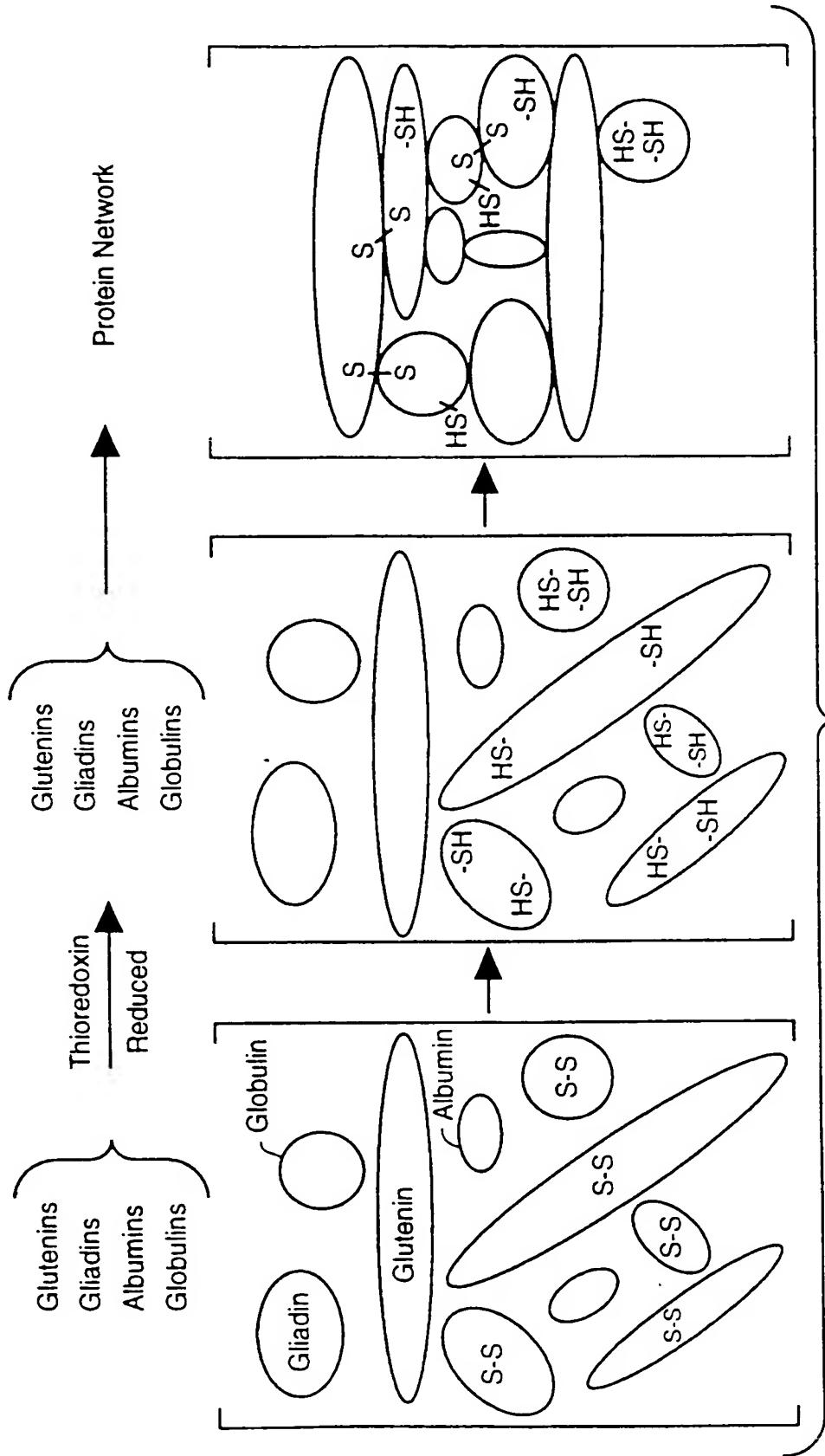


FIG. - 13

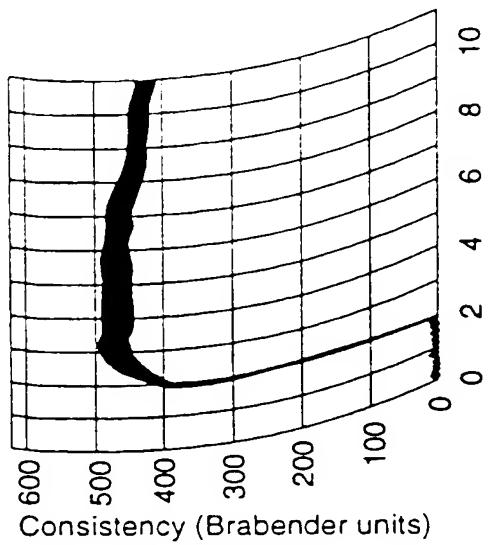


FIG. 14C

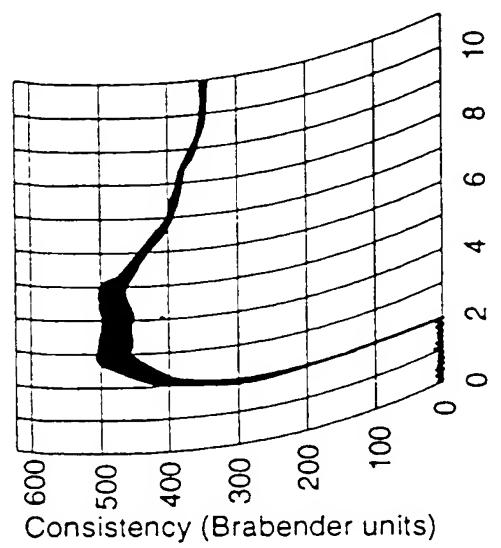


FIG. 14B

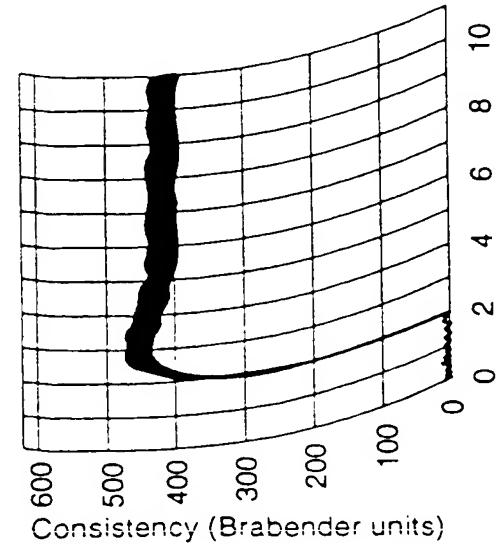
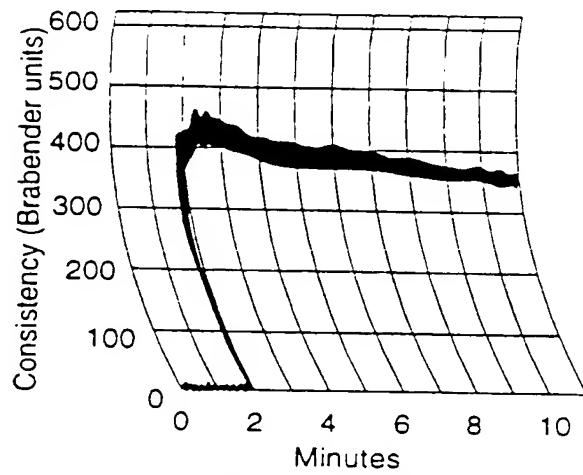
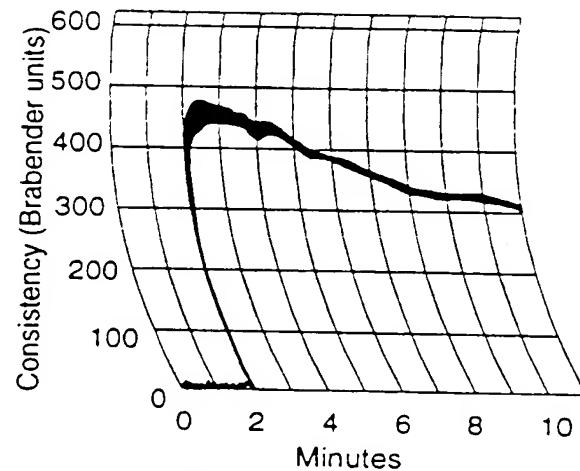


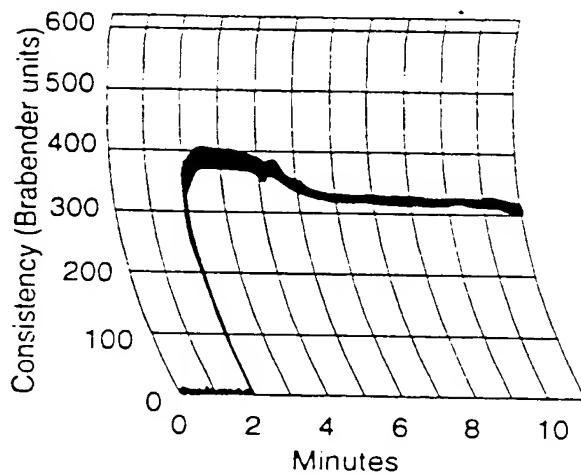
FIG. 14A



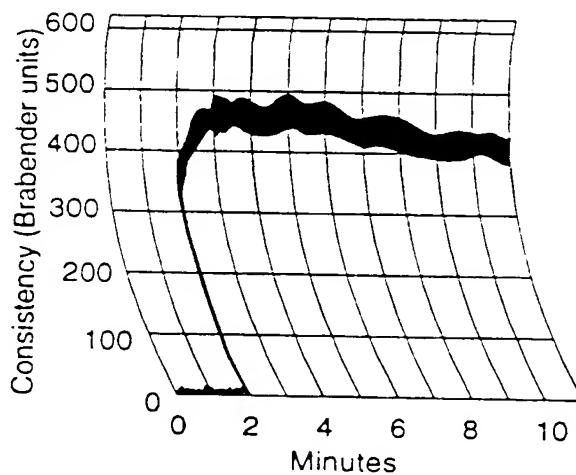
**FIG. 15A**



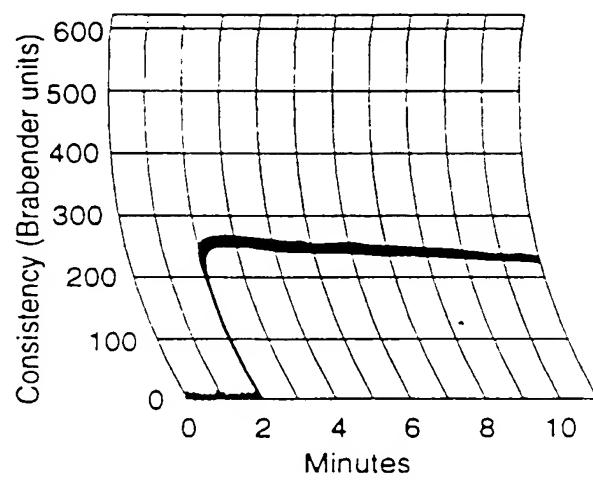
**FIG. 15B**



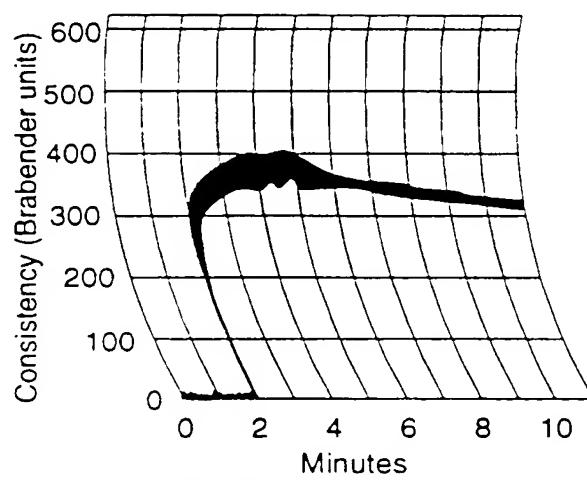
**FIG. 15C**



**FIG. 15D**



**FIG.\_ 16A**



**FIG.\_ 16B**

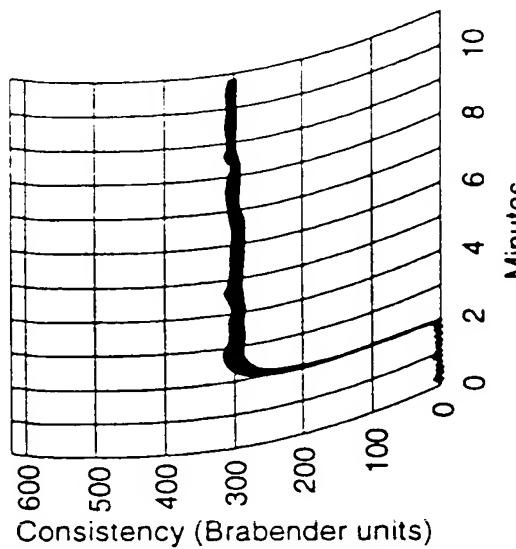


FIG.\_17C

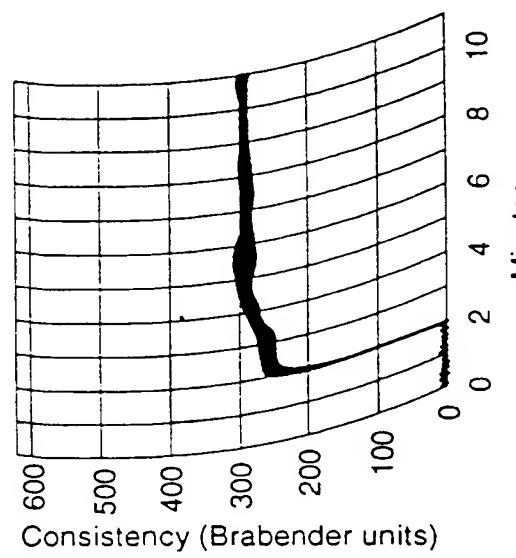


FIG.\_17B

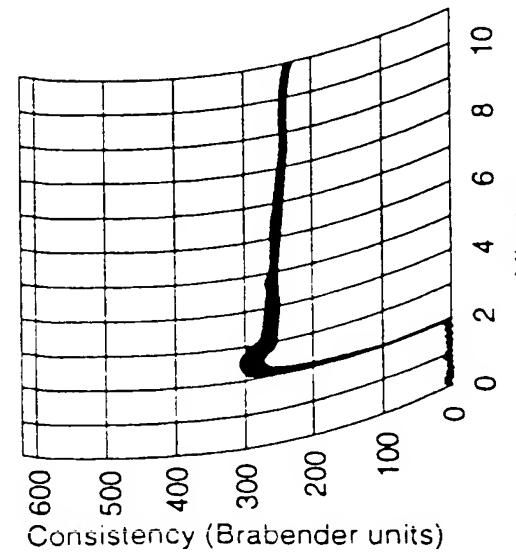


FIG.\_17A

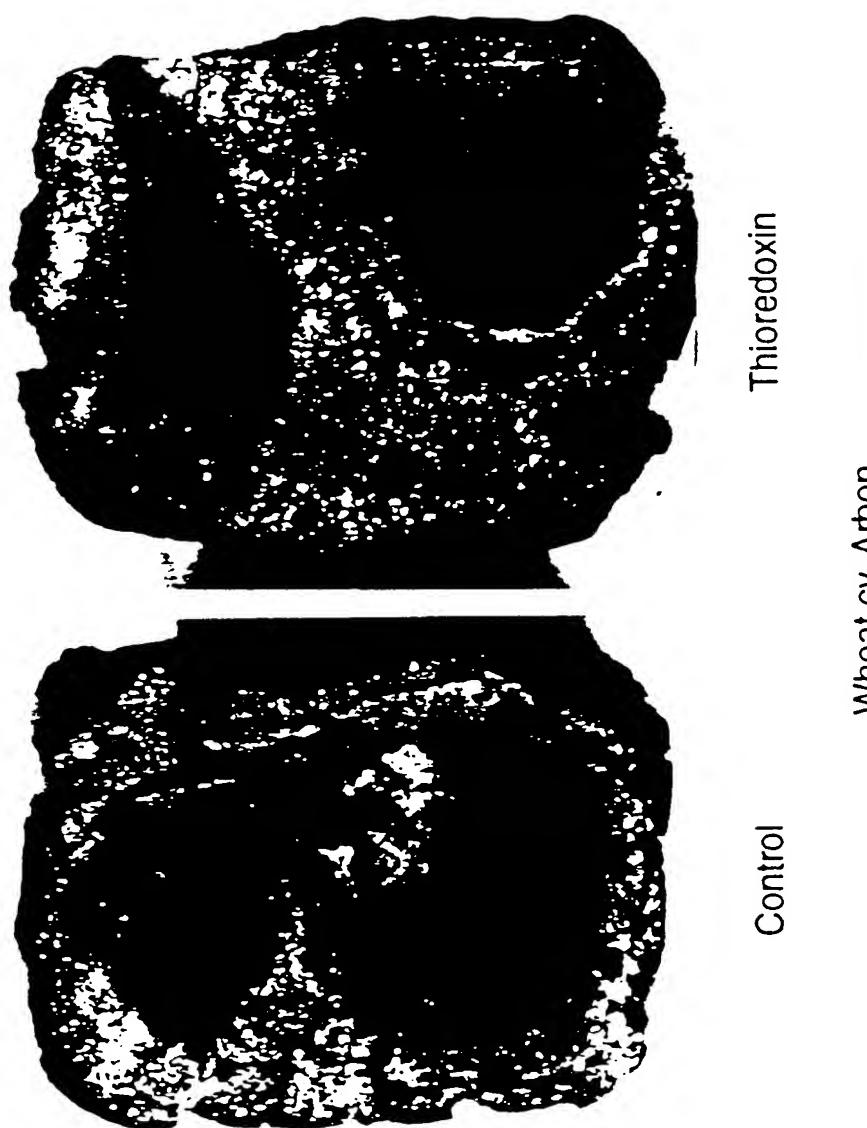
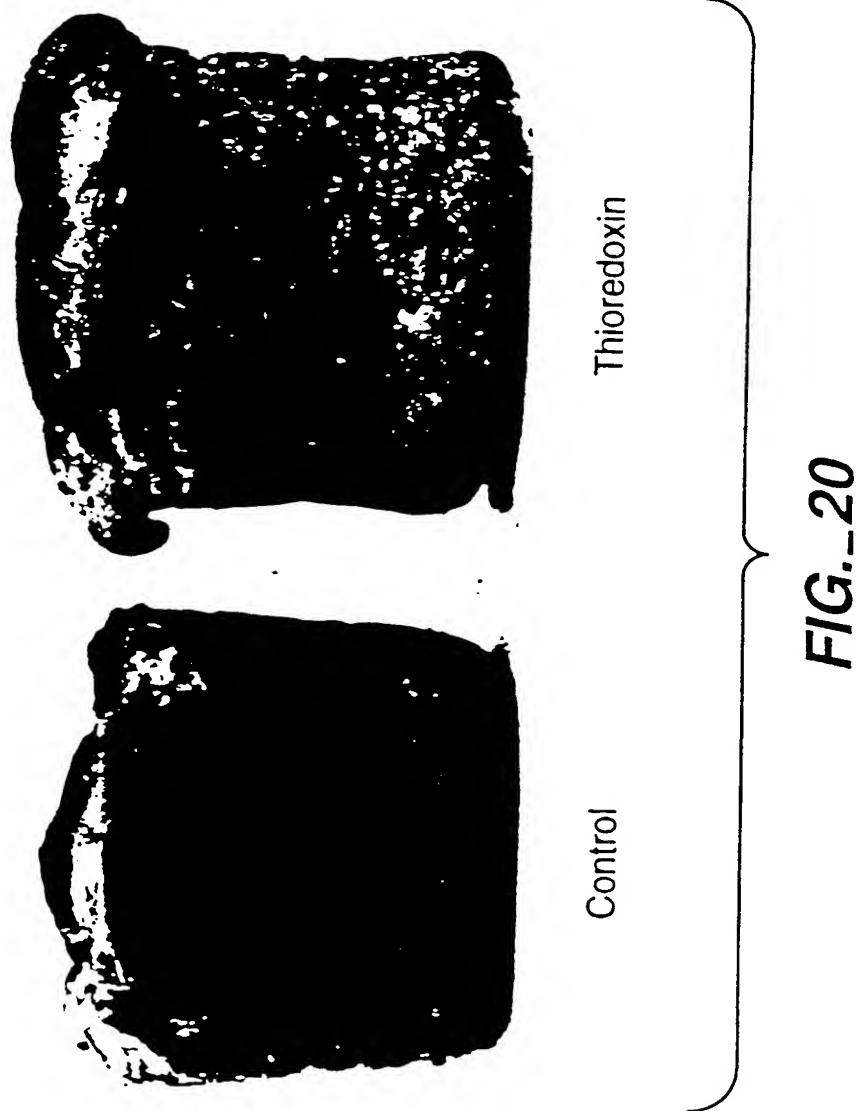


FIG.\_18





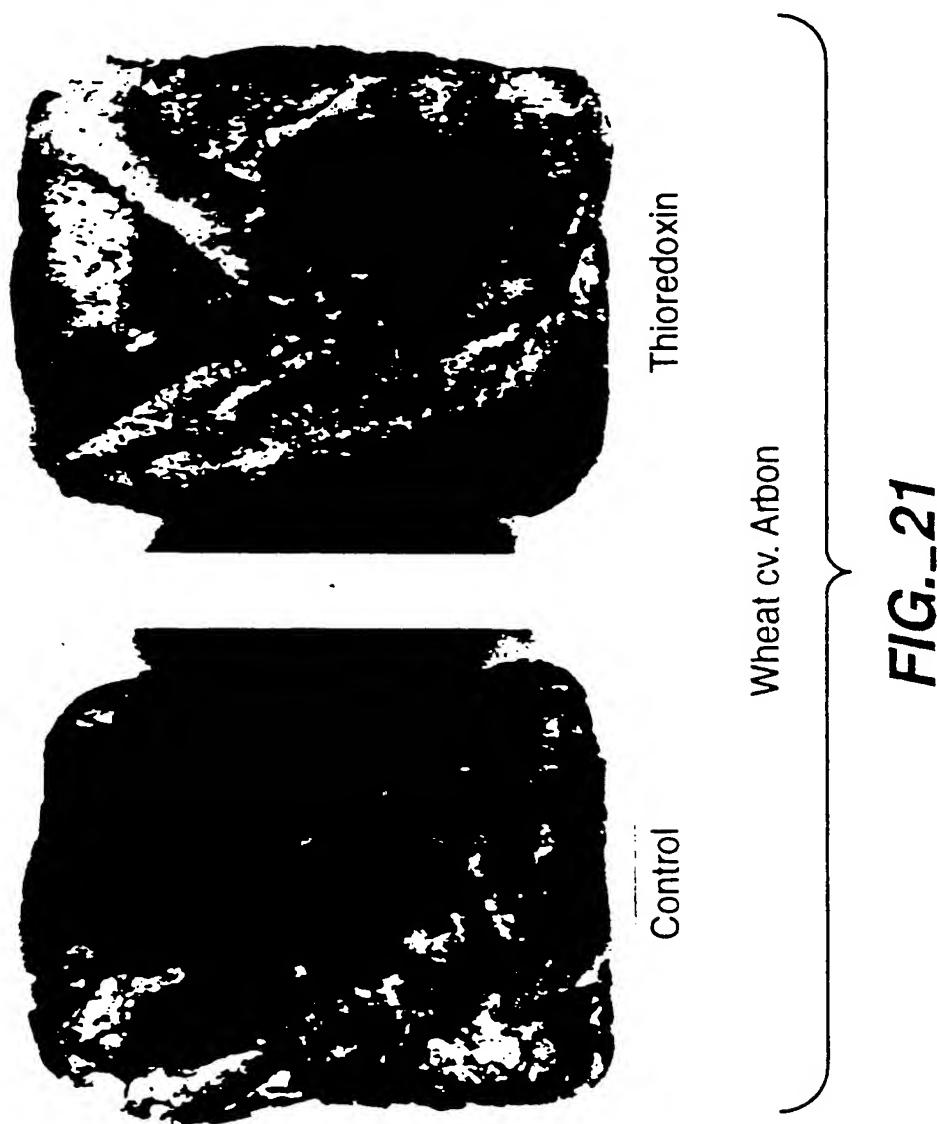


FIG. 21

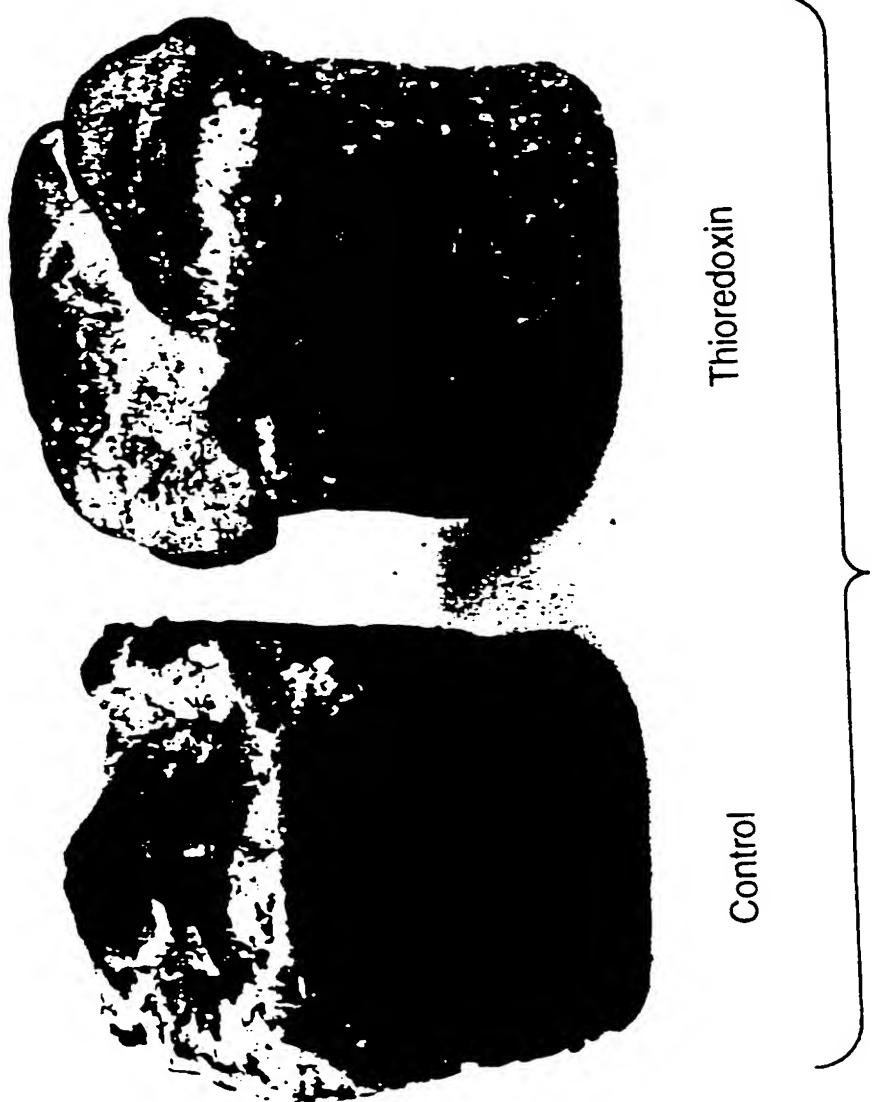


FIG.\_22

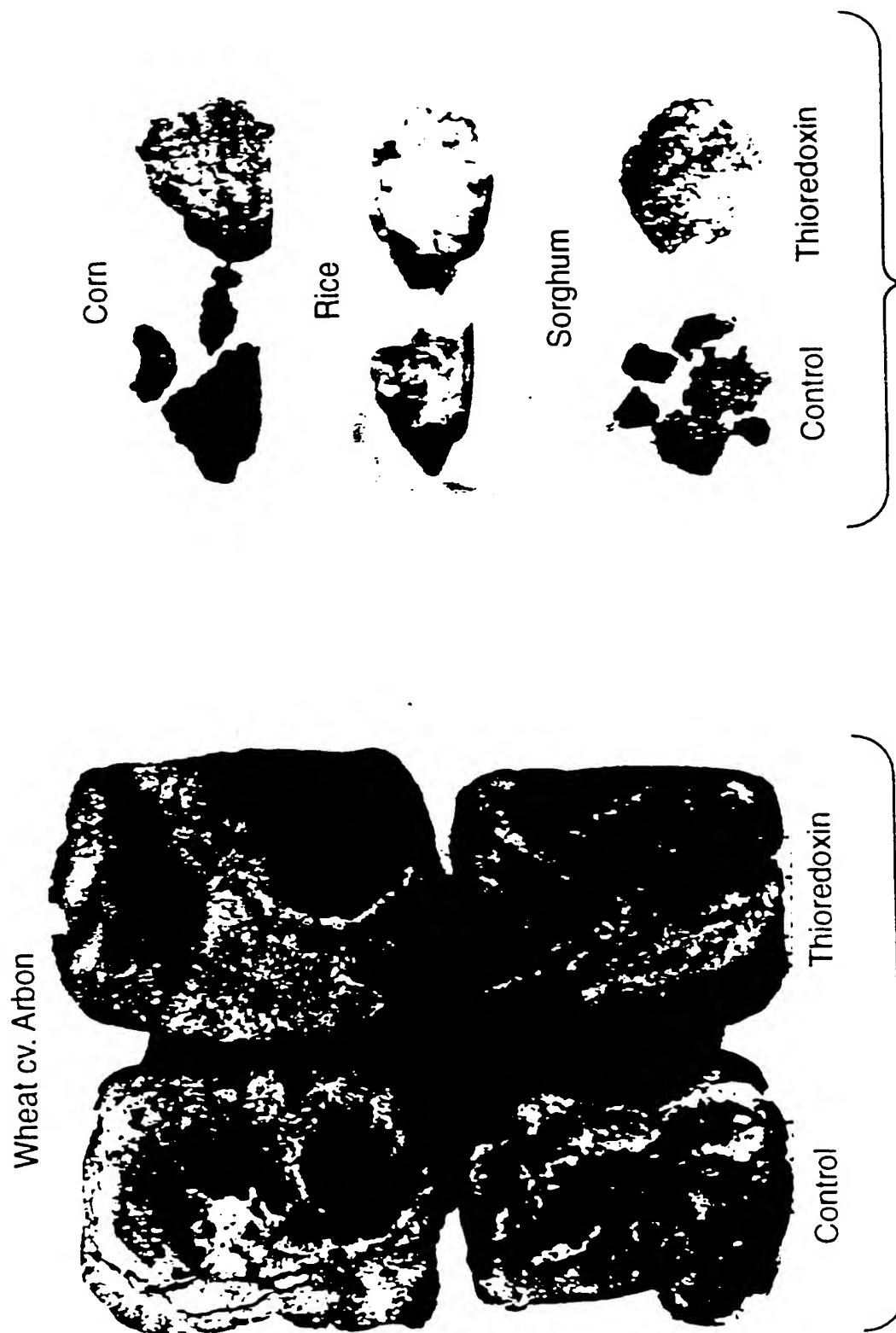
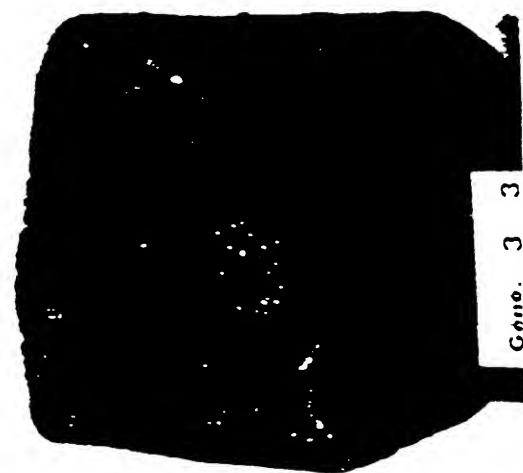


FIG. 23B

FIG. 23A



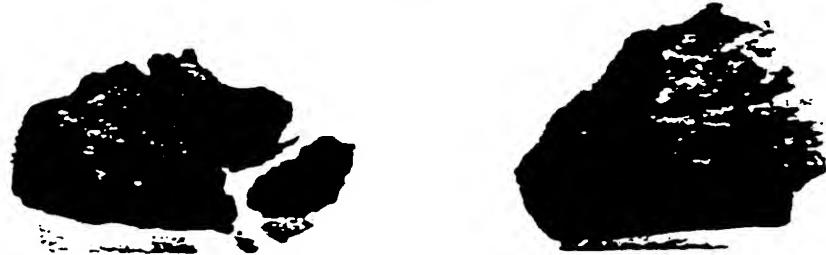
TÉMOIN



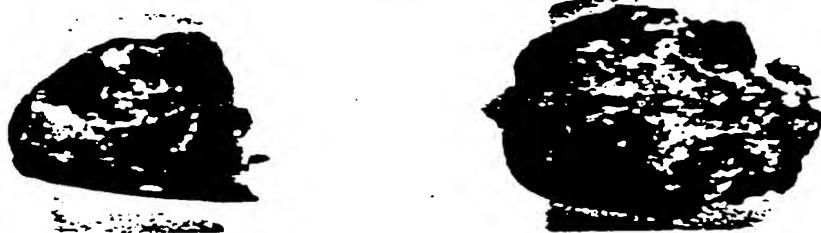
Géné. - 3 - 3

FIG. 24

Com



Rice



Sorghum

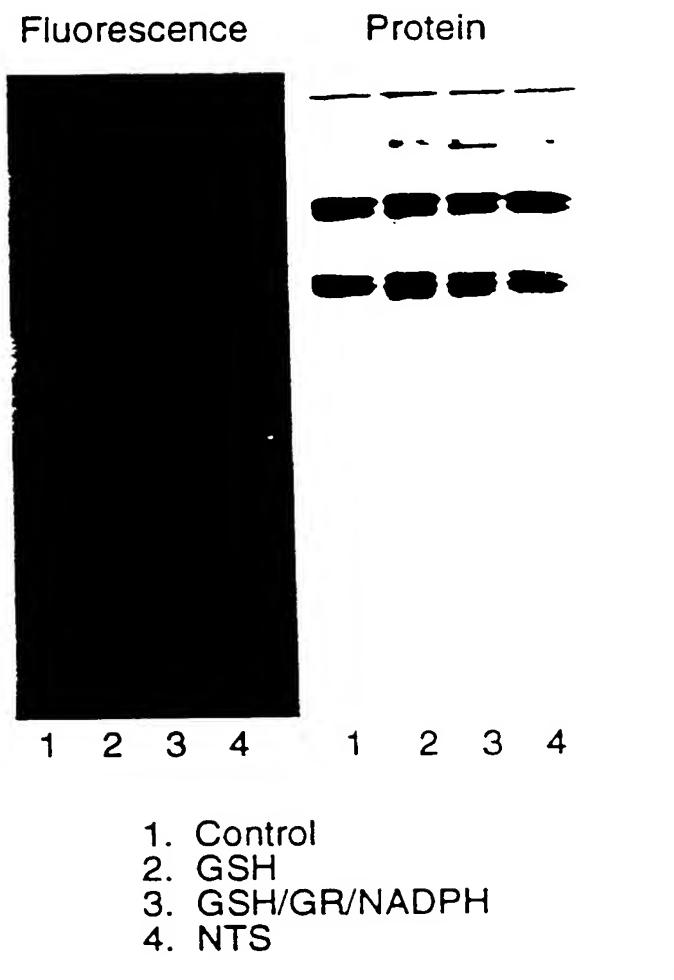


Control

Thioredoxin

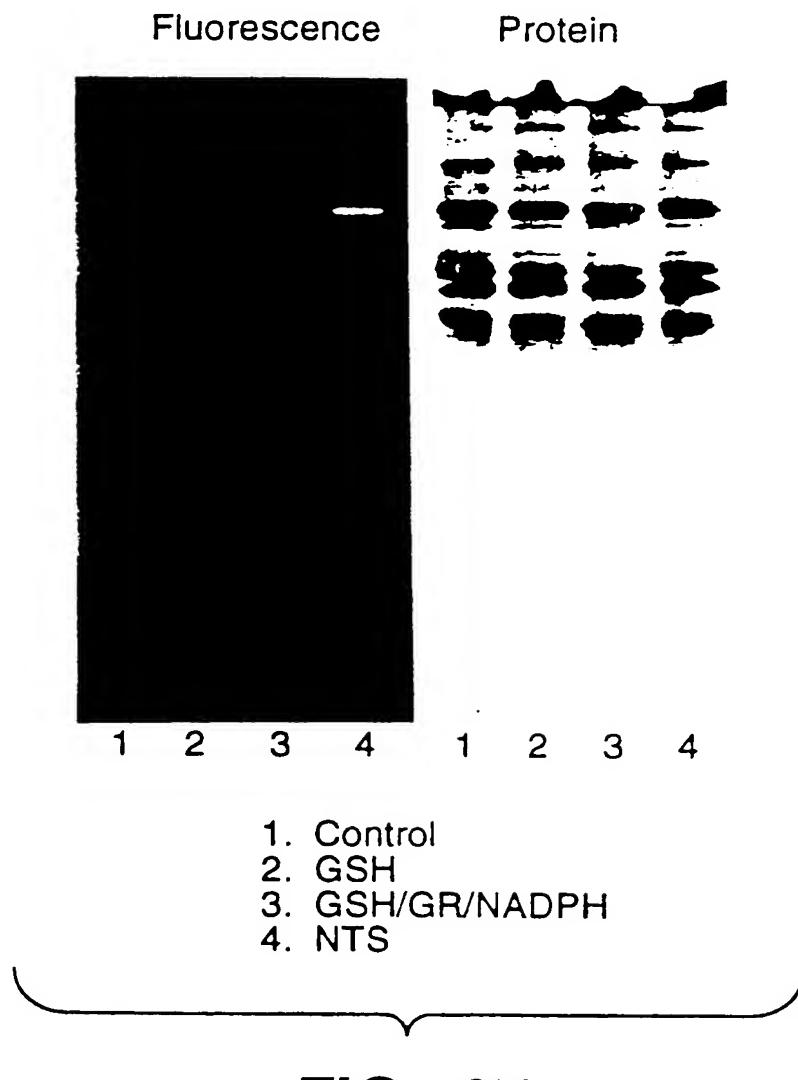
***FIG.\_25***

Thioredoxin-linked Reduction  
of Myristate-Extracted Proteins  
from Oat Flour

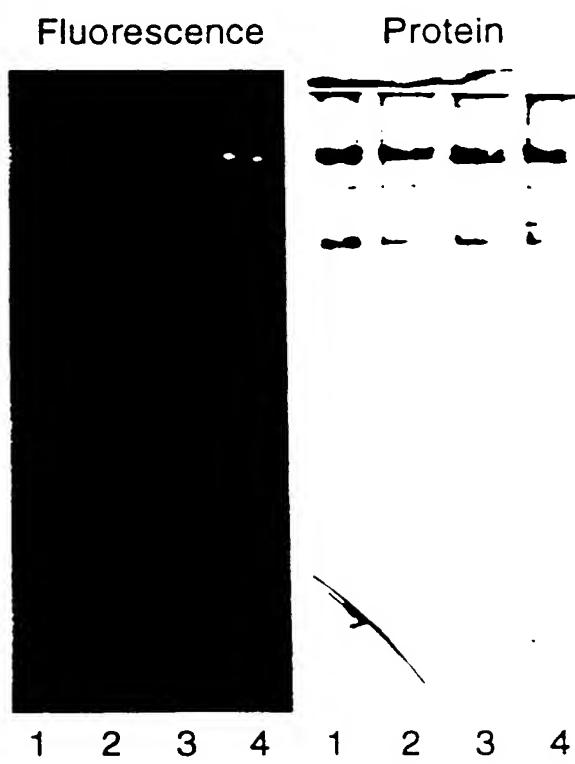


**FIG.\_26**

Thioredoxin-linked Reduction  
of Myristate-Extracted Proteins  
from Triticale Flour



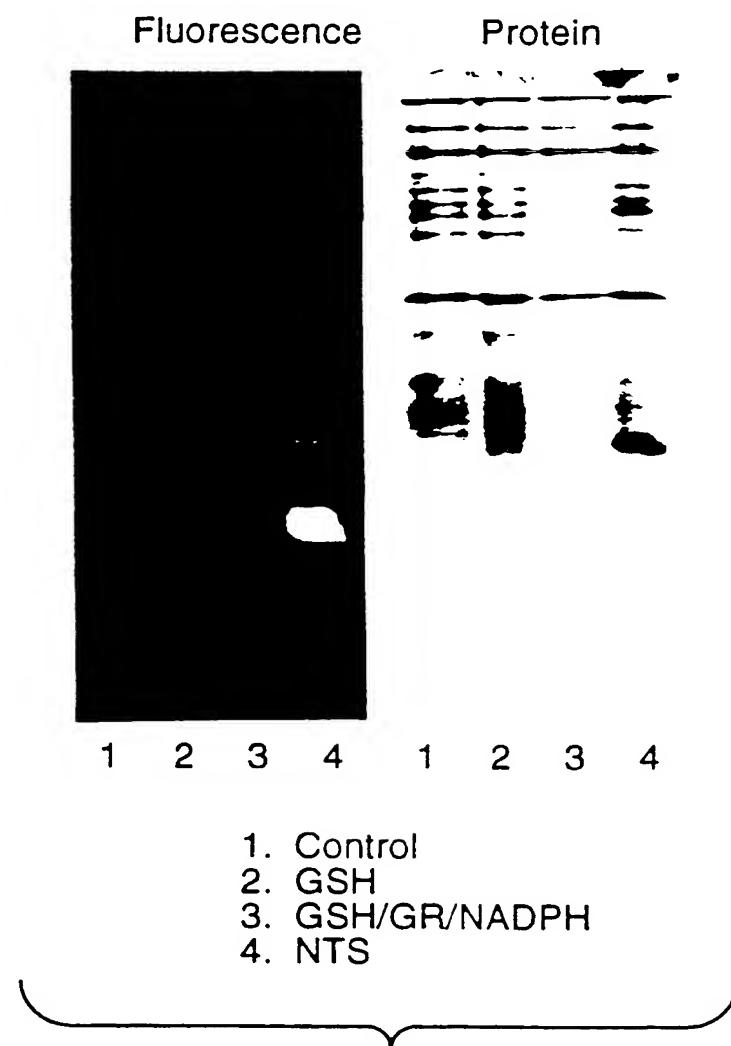
Thioredoxin-linked Reduction  
of Myristate-Extracted Proteins  
from Rye Flour



1. Control
2. GSH
3. GSH/GR/NADPH
4. NTS

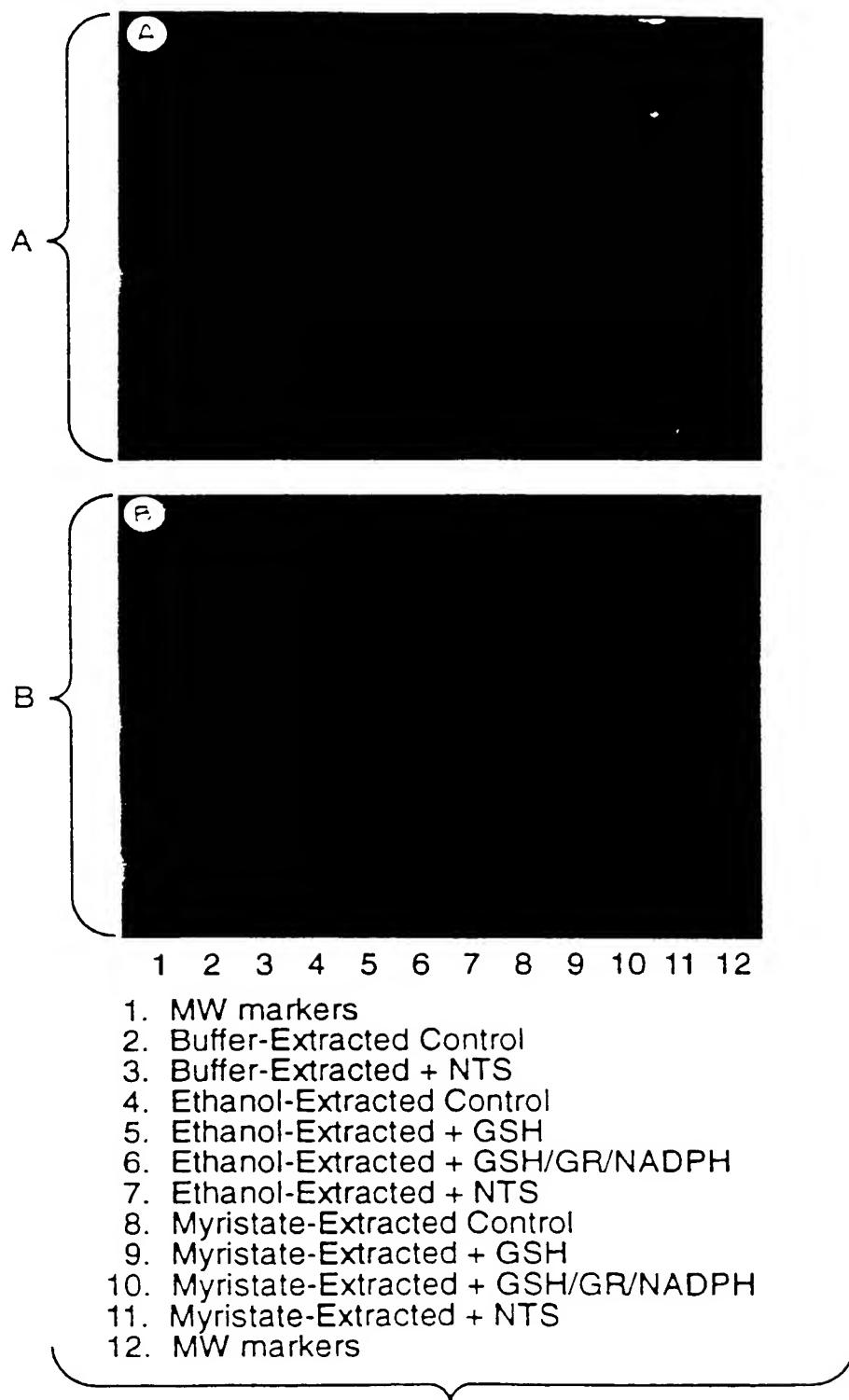
**FIG.\_28**

Thioredoxin-linked Reduction  
of Myristate-Extracted Proteins  
from Barley Flour



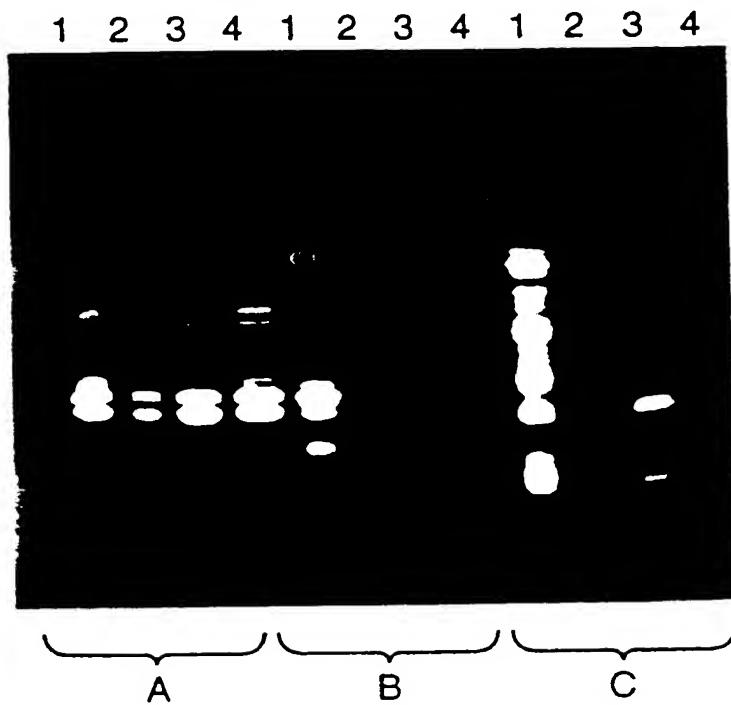
**FIG.\_29**

Thioredoxin-linked Reduction of Buffer-,  
Ethanol- and Myristate-Extracted Proteins  
from Teff (A) Fluorescence (B) Protein



**FIG.\_30**

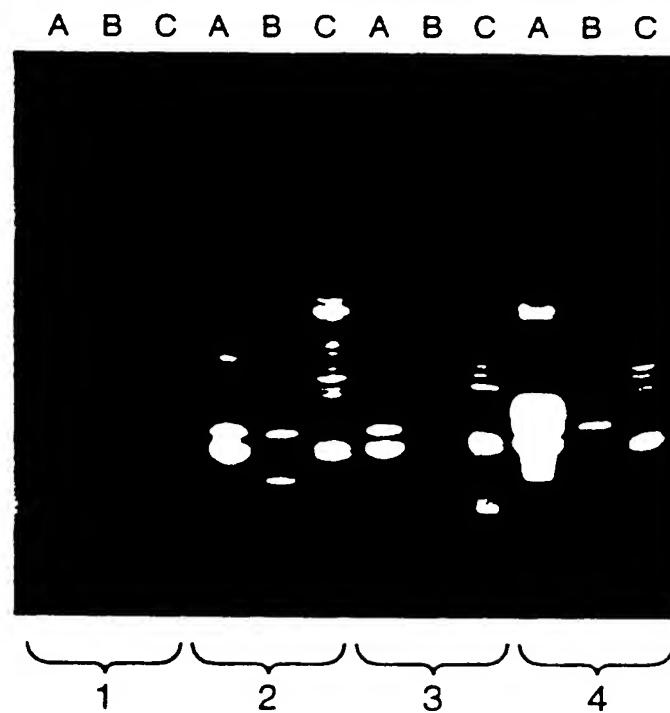
Effect of NTS vs Glutathione Reductase (GR) on  
Reduction Status of Myristate-Extracted Proteins  
from (A) Corn, (B) Sorghum and (C) Rice



1. Sample + myristate - mbbr
2. Sample + myristate - 120 min - mbbr
3. Sample + myristate - 120 min - NTS - mbbr
4. Sample + myristate - 120 min - GR - mbbr

**FIG.\_31**

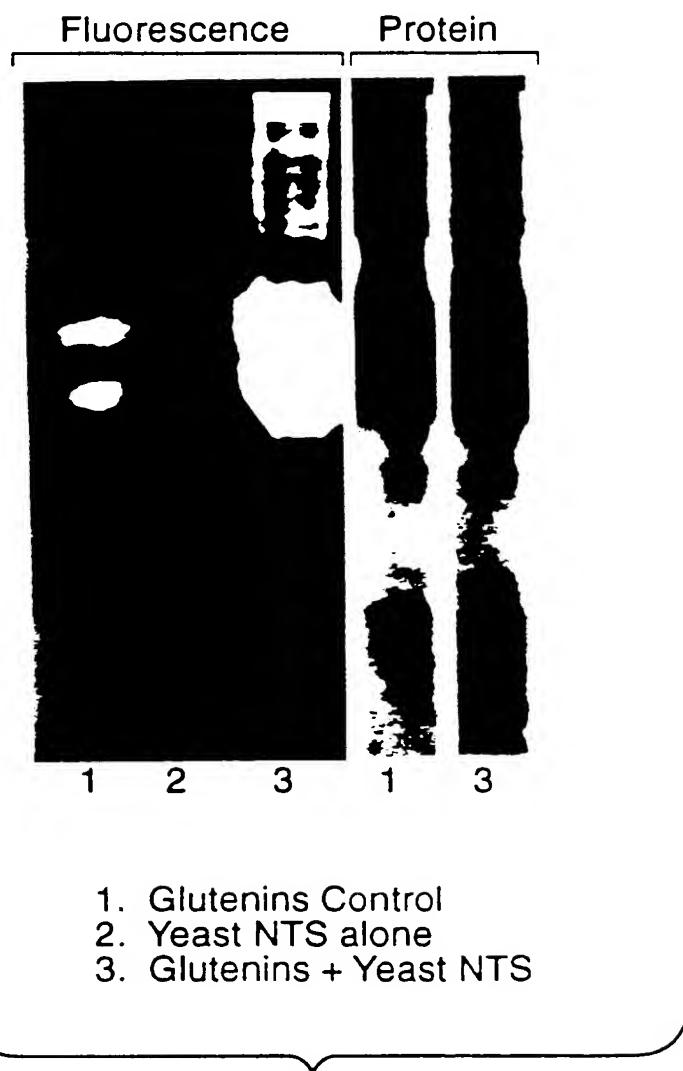
*In vivo* Reduction Status and Thioredoxin-linked  
*in vitro* Reduction of Myristate-Extracted Proteins  
from (A) Corn, (B) Sorghum and (C) Rice



1. Sample + myristate - 120 min - mbbr
2. Sample + myristate - mbbr
3. Sample + myristate - 120 min - NTS - mbbr
4. Sample + myristate - 120 min - DTT - mbbr

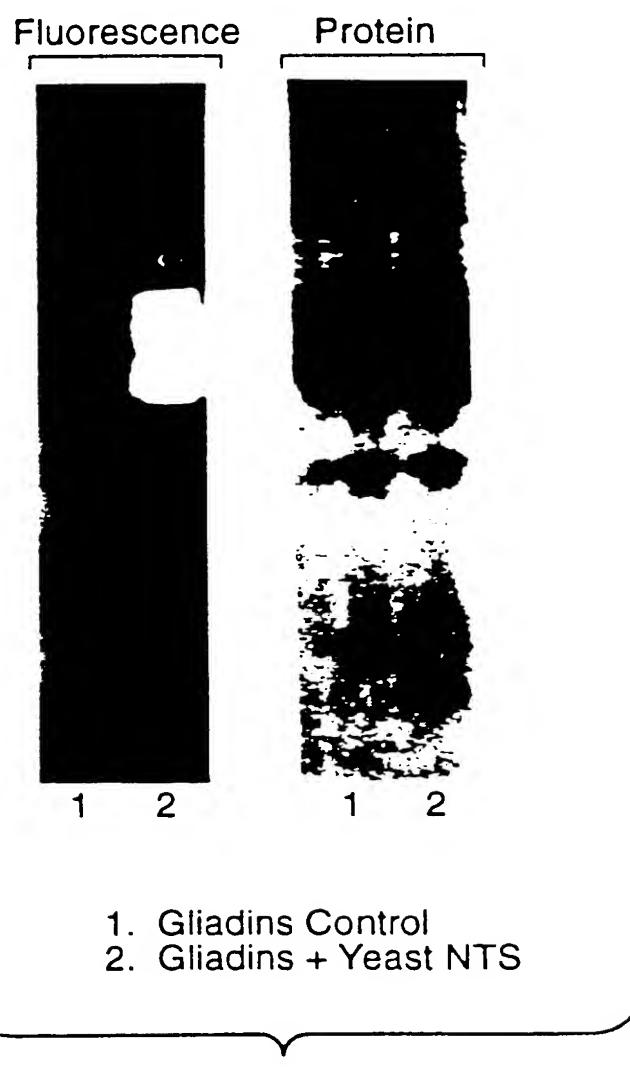
**FIG.\_32**

Relative Reduction of Wheat Glutenins  
by Yeast NADP/Thioredoxin System



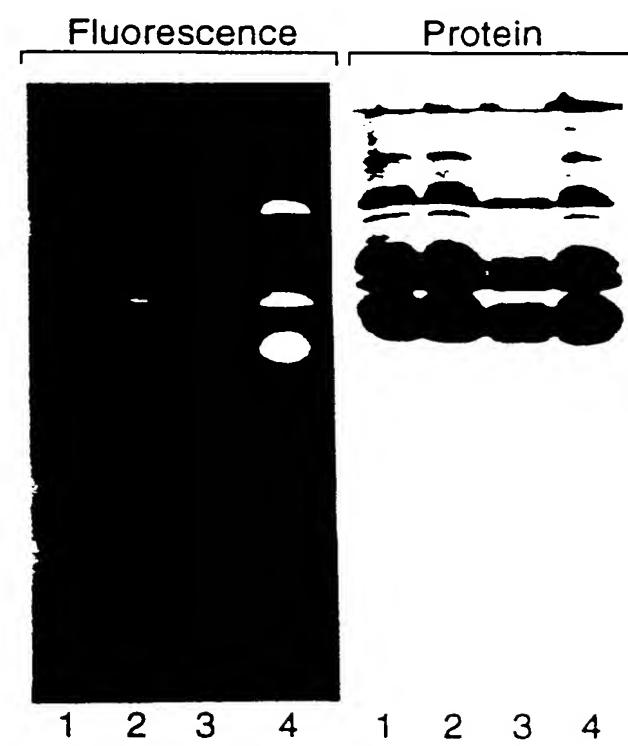
***FIG.\_33***

Relative Reduction of Wheat Gliadins  
by Yeast NADP/Thioredoxin System



**FIG.\_34**

Thioredoxin-linked Reduction  
of Ethanol-Extracted Proteins  
from Triticale Flour



- 1. Control
- 2. GSH
- 3. GSH/GR/NADPH
- 4. NTS

**FIG.\_35**

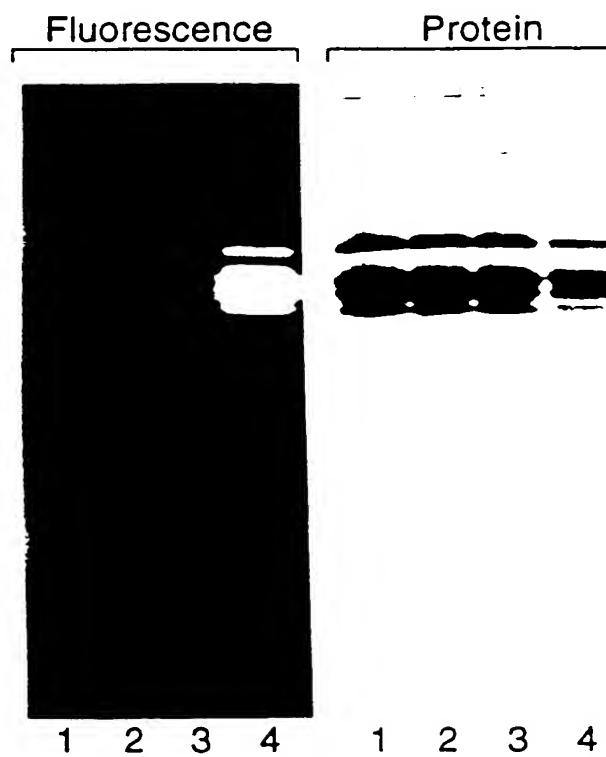
Thioredoxin-linked Reduction  
of Ethanol-Extracted Proteins  
from Rye Flour



1. Control
2. GSH
3. GSH/GR/NADPH
4. NTS

**FIG.\_36**

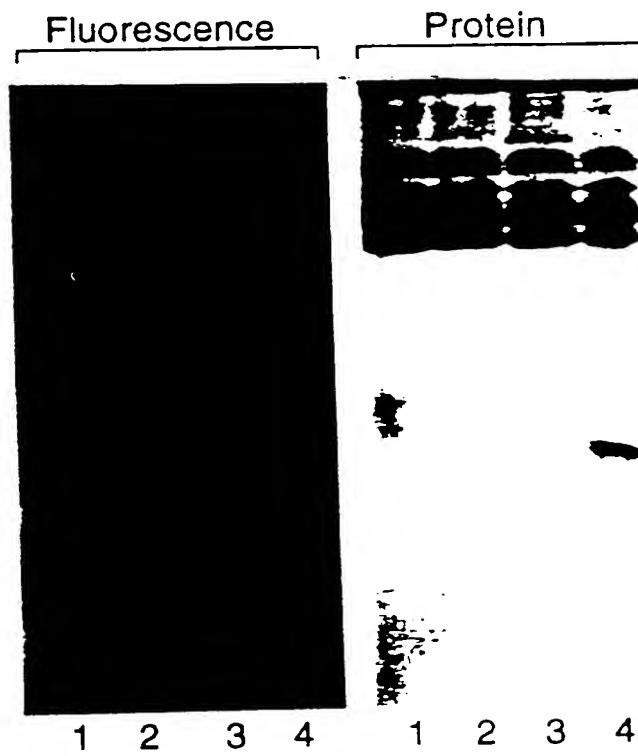
Thioredoxin-linked Reduction  
of Ethanol-Extracted Proteins  
from Oat Flour



1. Control
2. GSH
3. GSH/GR/NADPH
4. NTS

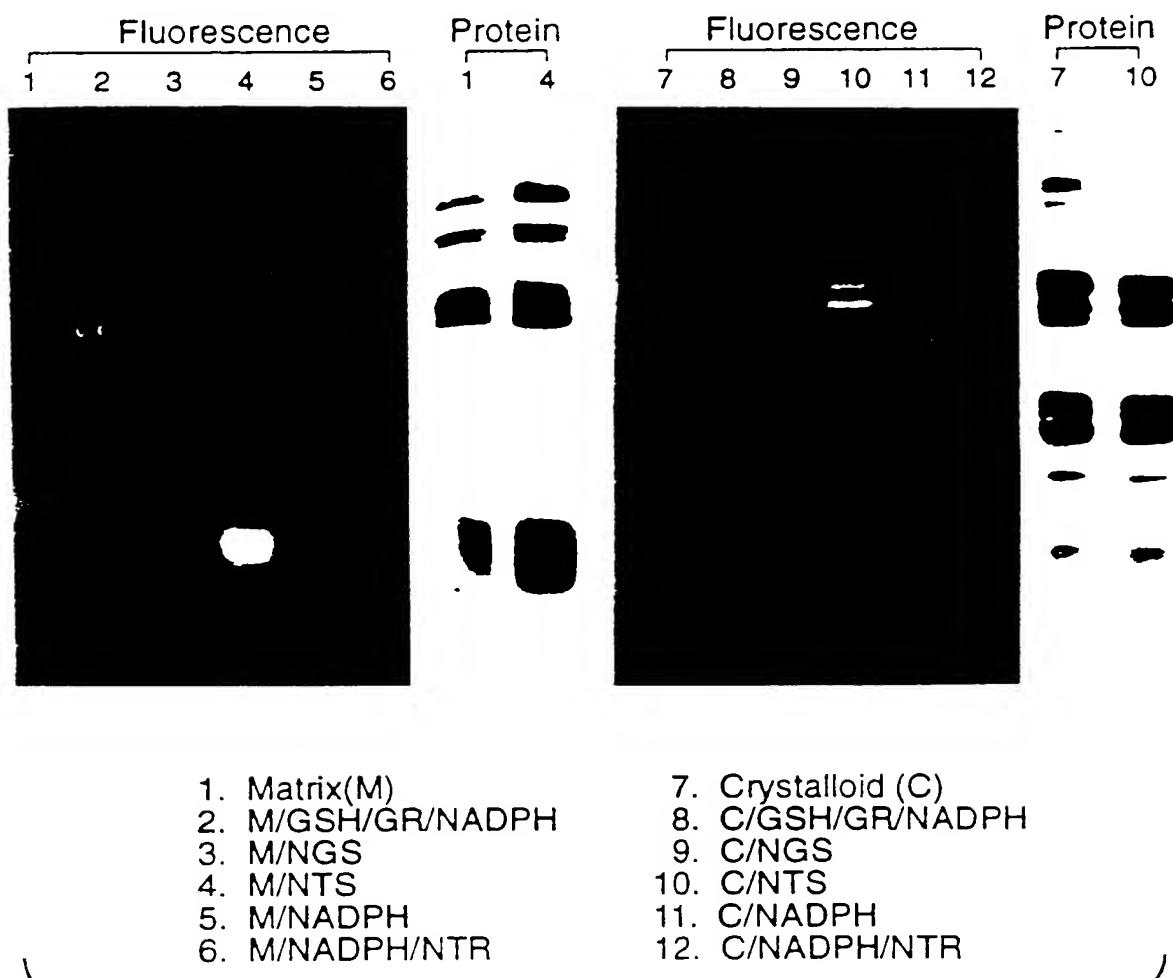
**FIG.\_37**

Thioredoxin-linked Reduction  
of Ethanol-Extracted Proteins  
from Barley Flour

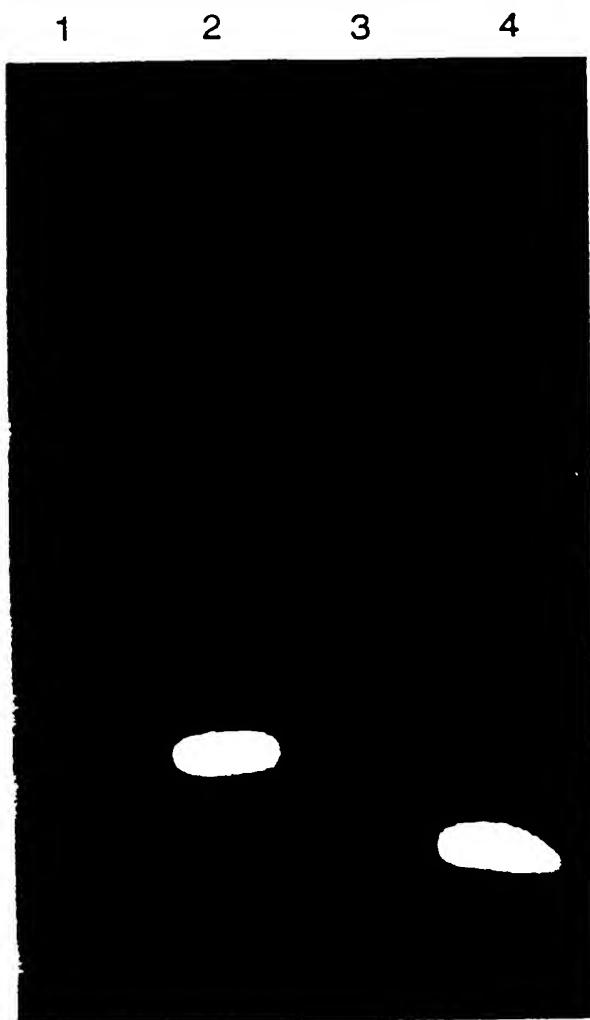


- 1. Control
- 2. GSH
- 3. GSH/GR/NADPH
- 4. NTS

**FIG. 38**



**FIG.\_39**



1. Control
2. Control + NTS
3. Control, 3 min Heat
4. Control + DTT, 3 min Heat

***FIG.\_40***

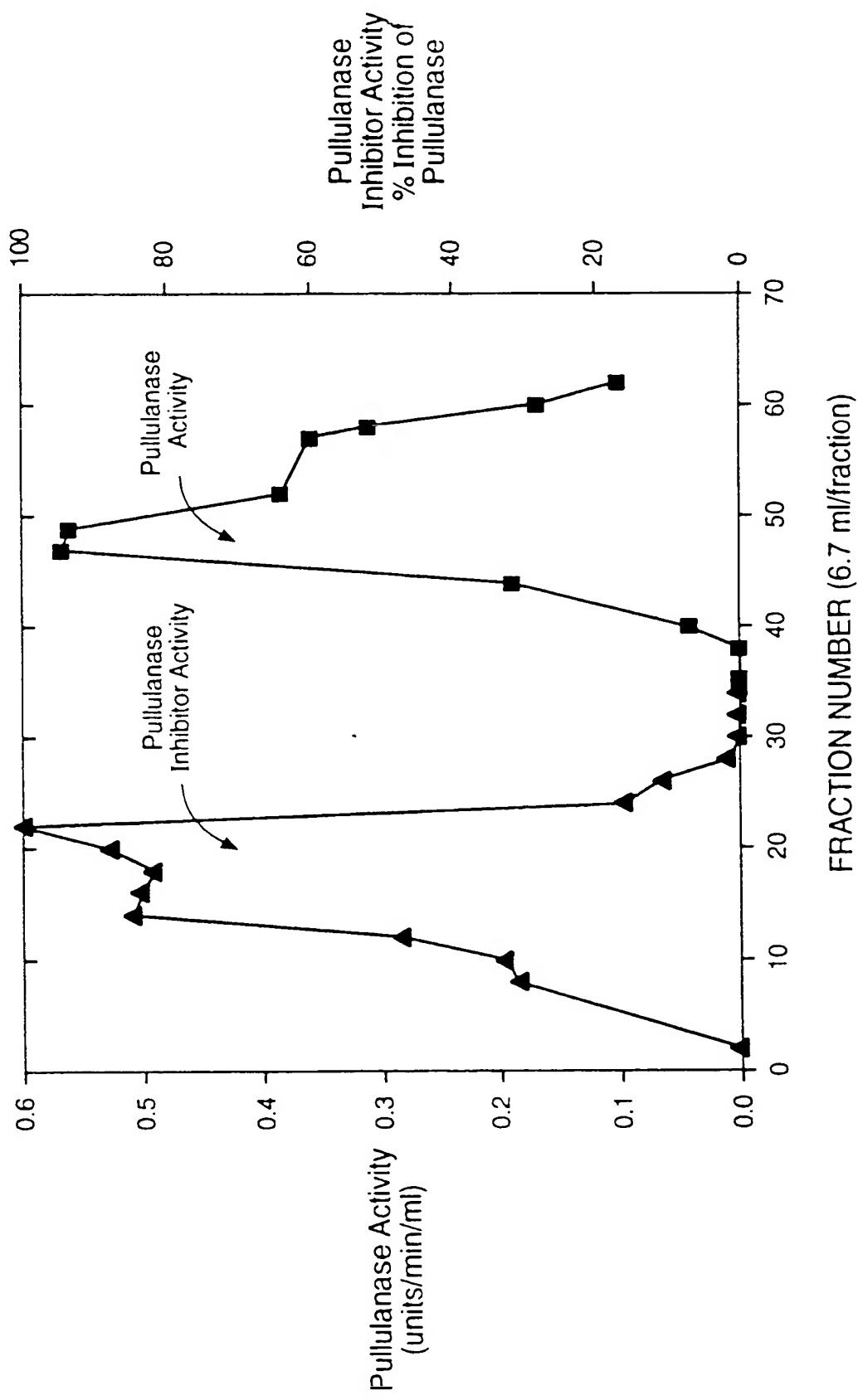


FIG. 41

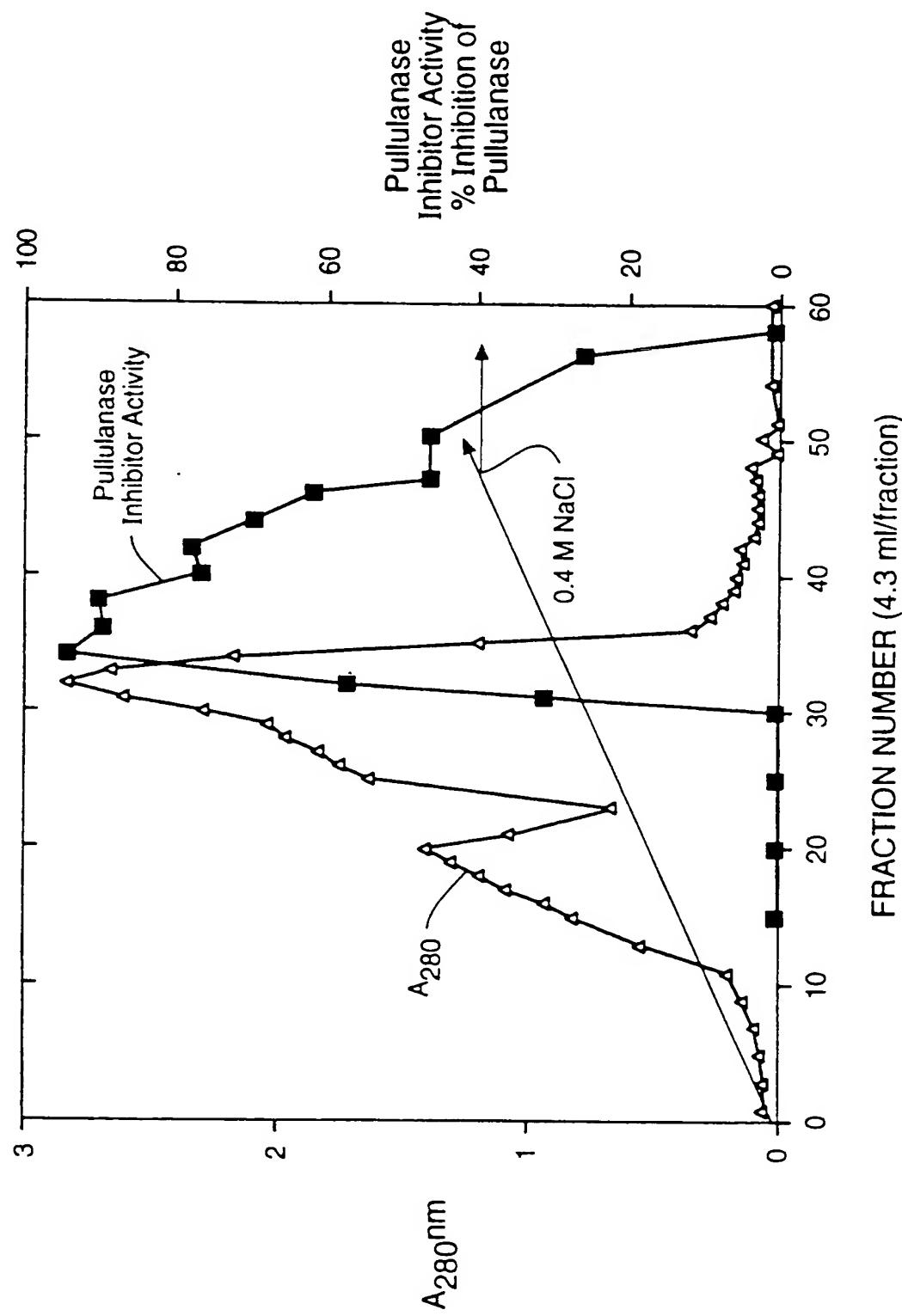


FIG. 42

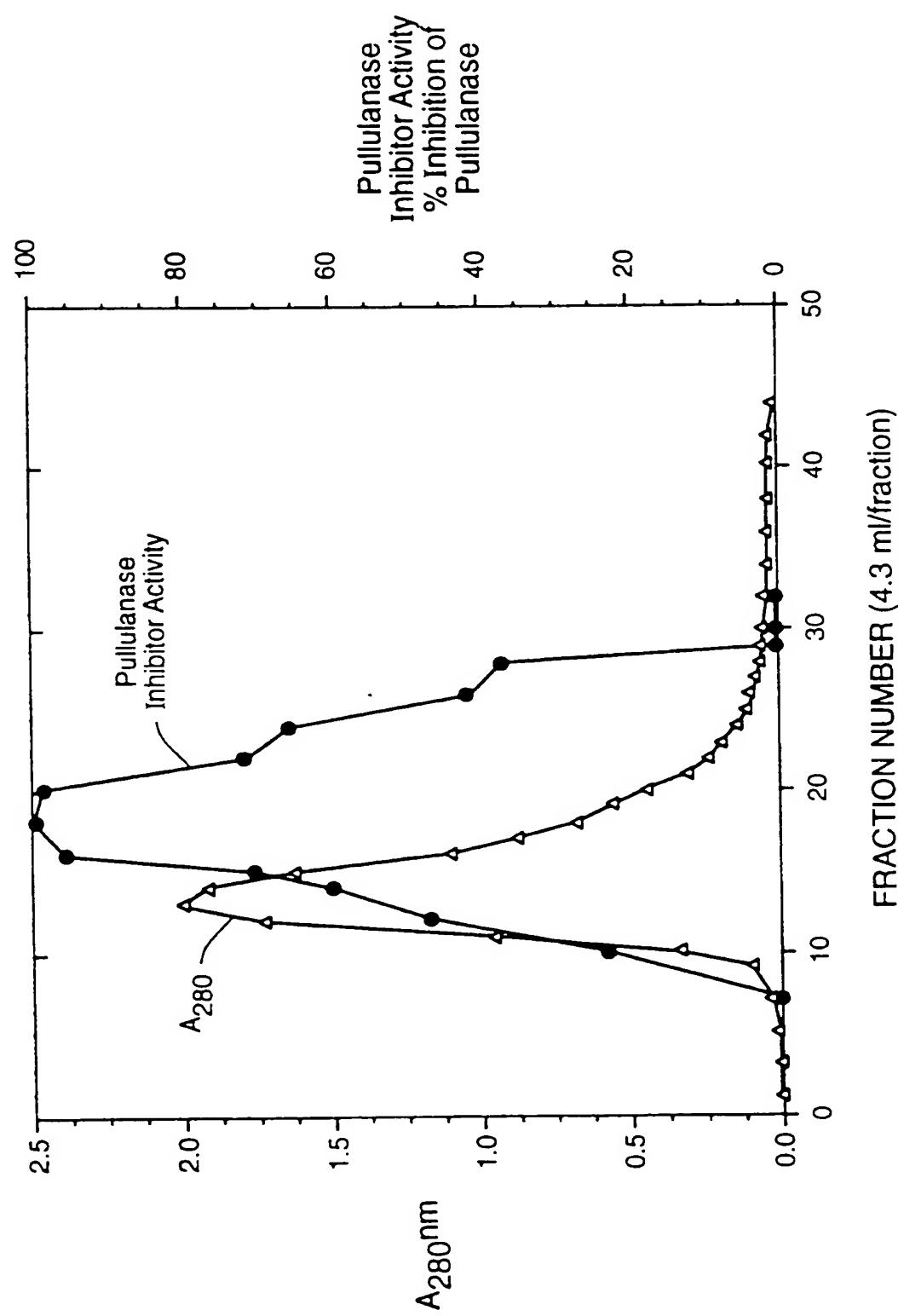


FIG. 43

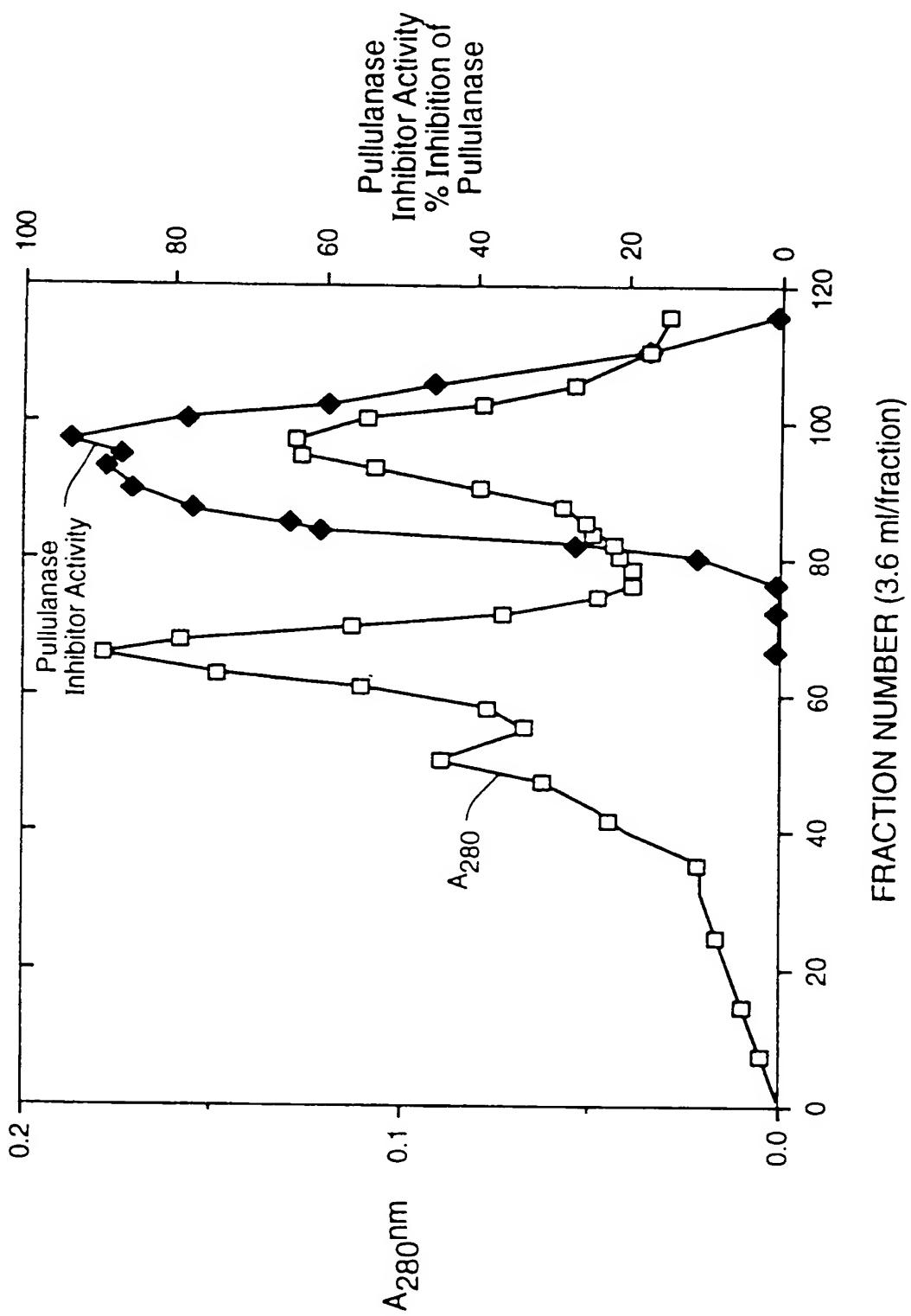
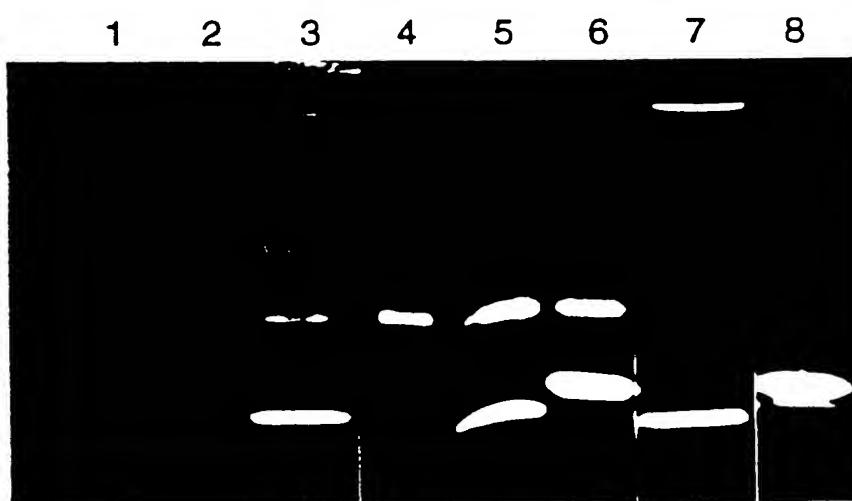


FIG. 44

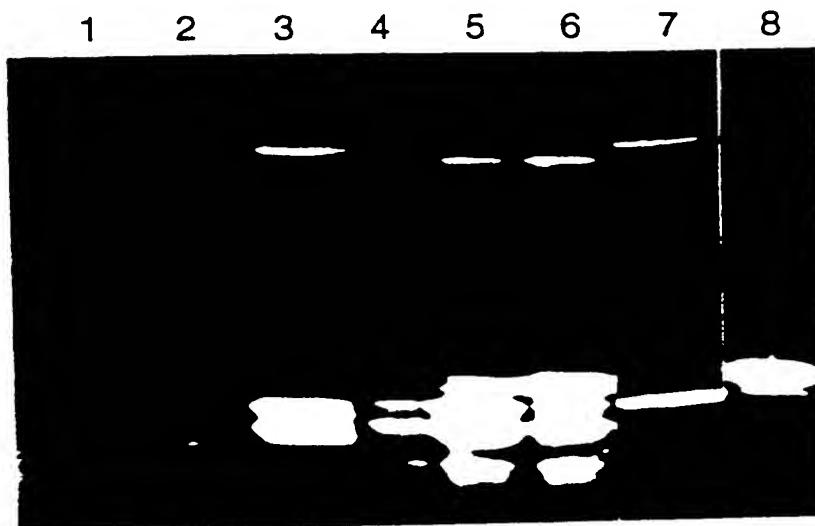
BEE VENOM FROM Apis mellifera



1. Control
2. GSH/NADPH, Glutathione reductase
3. NTS
4. DTT
5. DTT + *E. coli* Thioredoxin
6. DTT + Human Thioredoxin
7. NTS, no venom
8. DTT + Human Thioredoxin, no venom

**FIG.\_45**

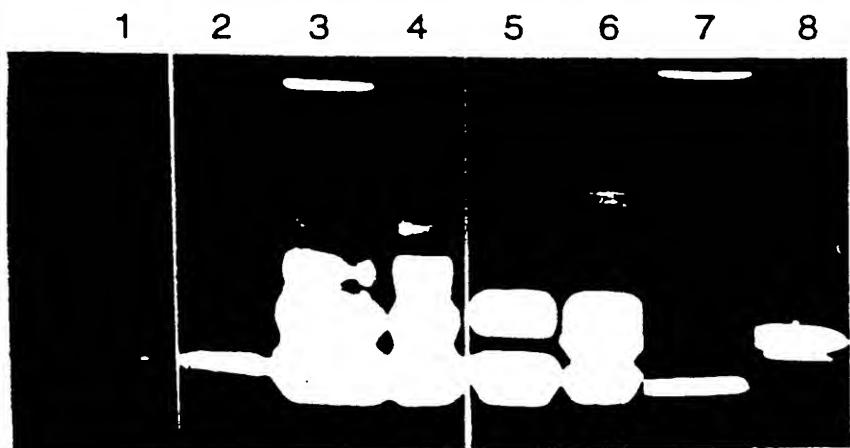
SCORPION VENOM FROM Androctonus australis



1. Control
2. GSH/NADPH/ Glutathione reductase
3. NTS
4. DTT
5. DTT + E. coli Thioredoxin
6. DTT + Human Thioredoxin
7. NTS, no venom
8. DTT + Human Thioredoxin, no venom

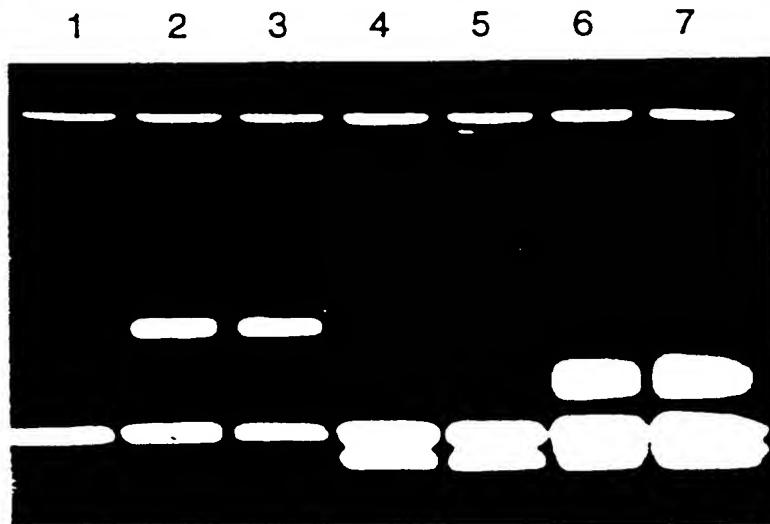
**FIG.\_46**

SNAKE VENOM FROM Bungarus multicinctus



1. Control
2. GSH/NADPH/ Glutathione reductase
3. NTS
4. DTT
5. DTT + E. coli Thioredoxin
6. DTT + Human Thioredoxin
7. NTS, no venom
8. DTT + Human Thioredoxin, no venom

**FIG.\_47**



1. NADP – Thioredoxin System
2. Bee Venom + NTS + Protease inhibitors
3. Bee Venom + NTS – Protease inhibitors
4. Scorpion Venom + NTS + Protease inhibitors
5. Scorpion Venom + NTS – Protease inhibitors
6. Snake Venom + NTS + Protease inhibitors
7. Snake Venom + NTS – Protease inhibitors

***FIG.\_48***

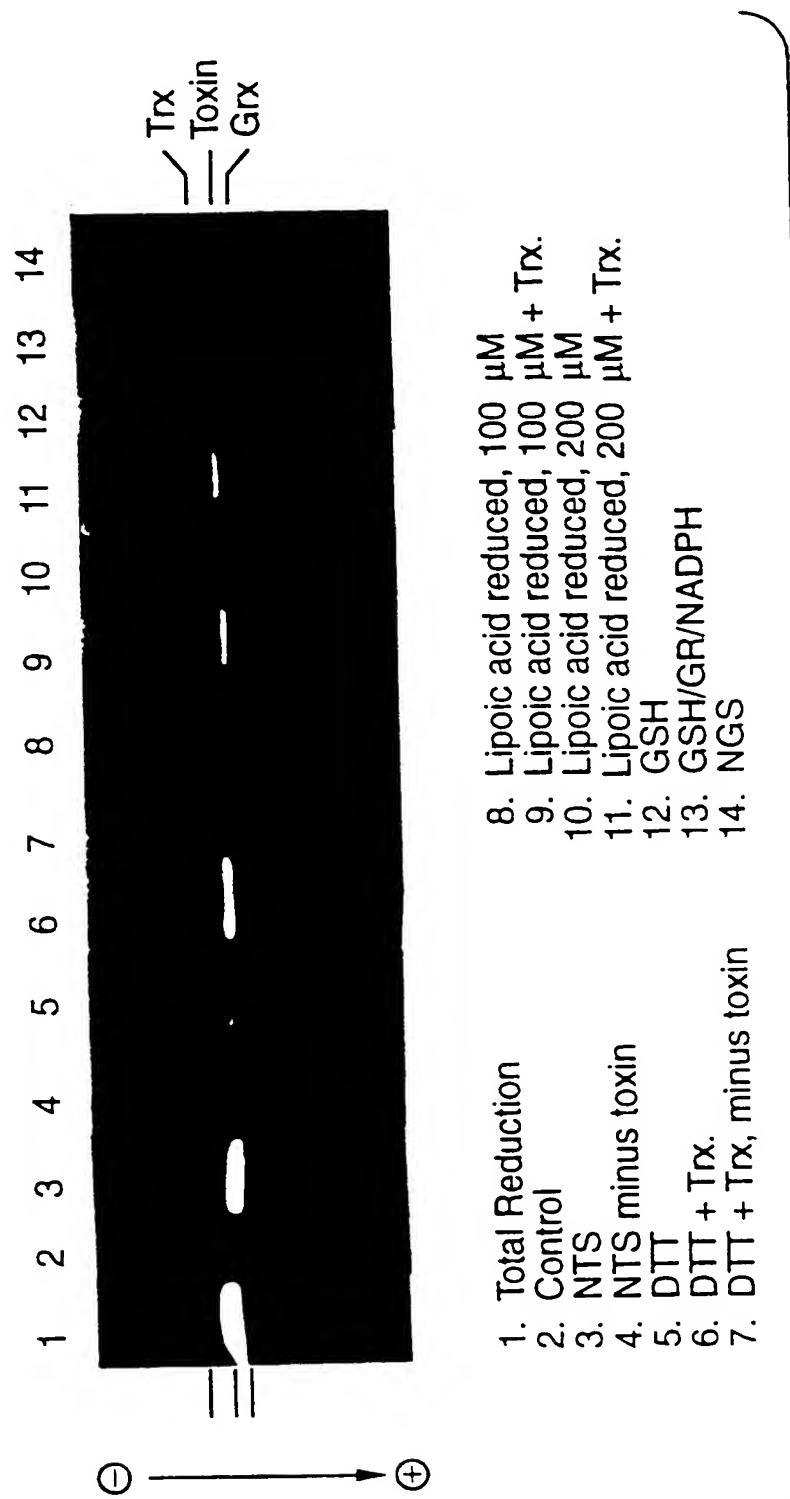


FIG. 49

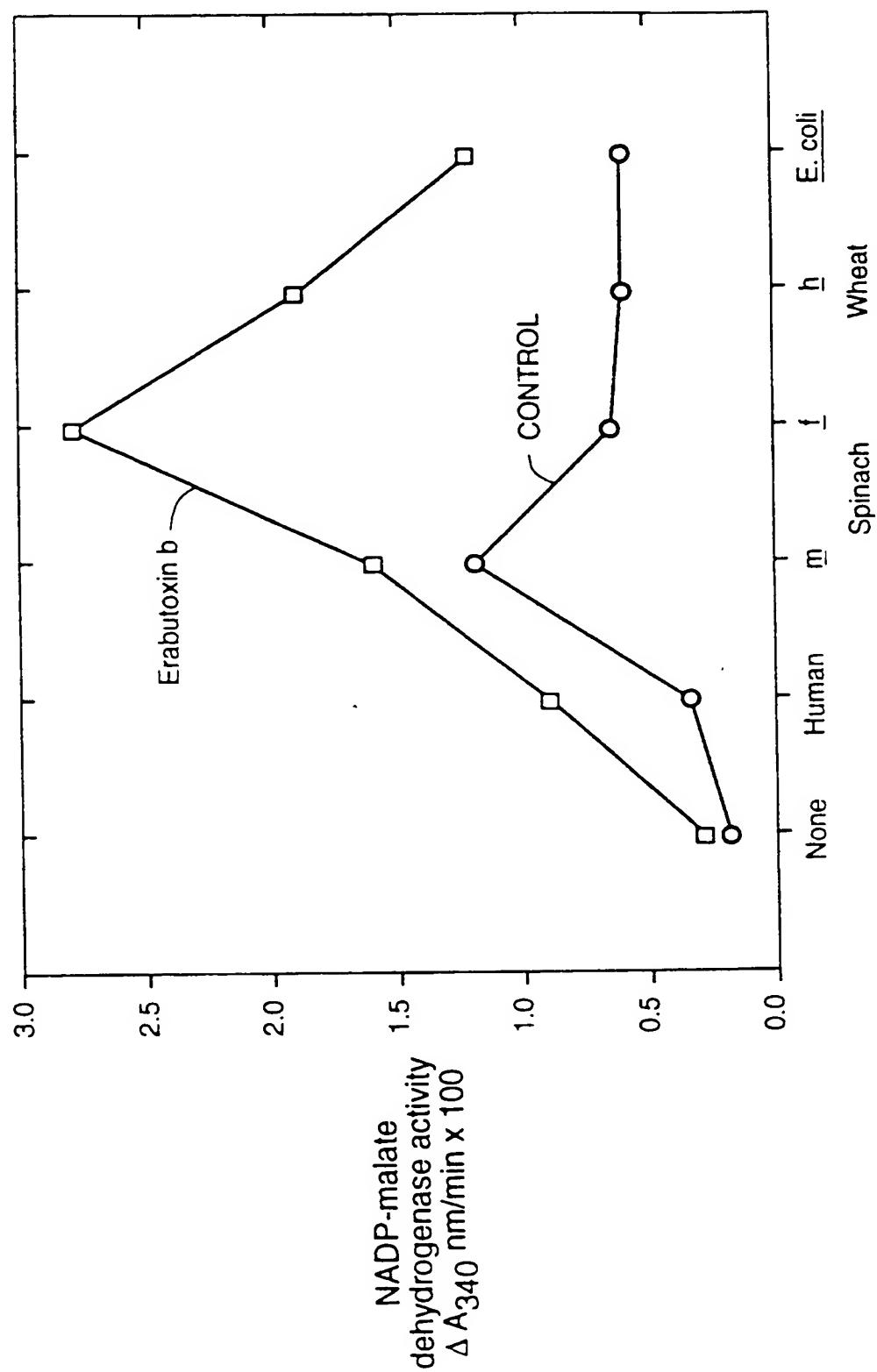


FIG. 50

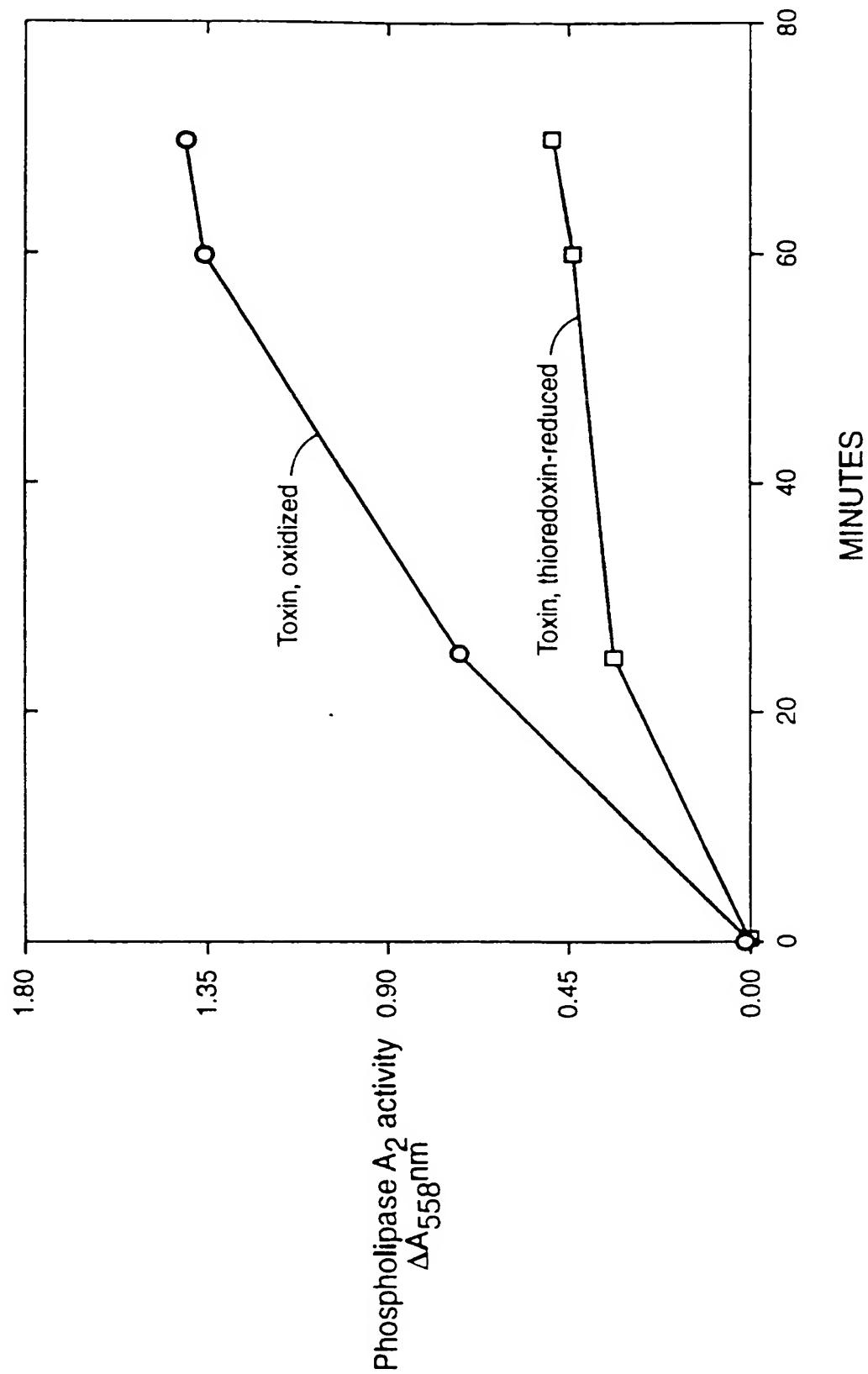


FIG. 51

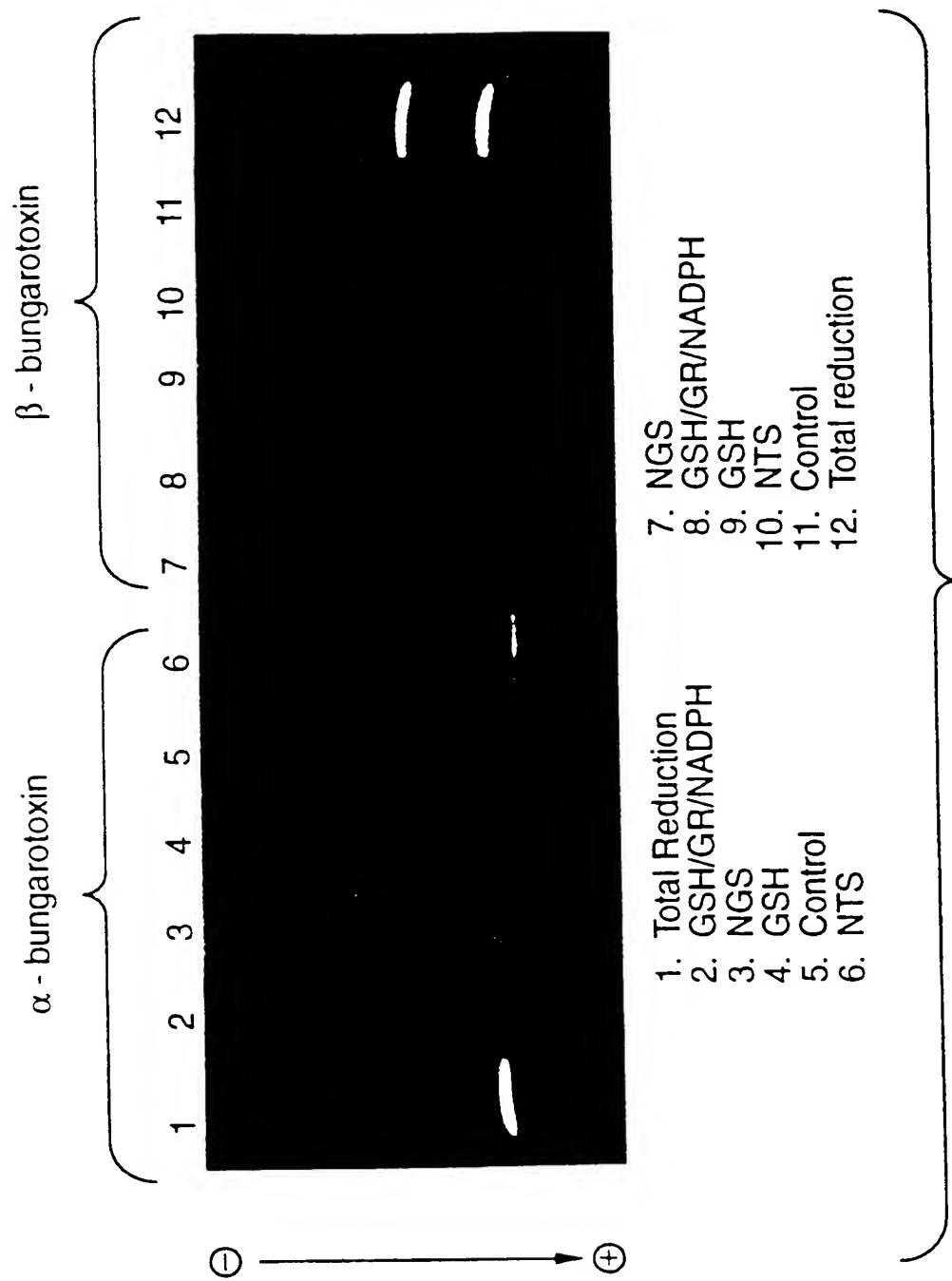


FIG. 52